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THE BACTERIOLOGY OF CEREBRO-SPINAL MENINGITIS.

*Opening Paper read in the Section of Pathology and Bacteriology
at the Annual Meeting of the British Medical Association,
Cambridge, July 2nd, 1920.*

By JOSEPH A. ARKWRIGHT, M.D., F.R.C.P.,
ASSISTANT BACTERIOLOGIST, LISTER INSTITUTE OF PREVENTIVE MEDICINE.

THE epidemic of cerebro-spinal meningitis during the war has produced an enormous amount of careful bacteriological work. Whatever its immediate practical value and the fate of the conclusions drawn from the data by the different workers, much of the work itself has been extraordinarily thorough and accurate, and must have a value in elucidating the general problems of the classification of bacteria, their liability to change, and the relation of pathogenic bacteria to their nearly related non-pathogenic congeners. The close study of the occurrence, position and persistence of meningococci in the nasopharynx of carriers will also be of general value as an example of the relation of bacterial infection to the course of epidemics of disease and as affording facts concerning the spread of an air-borne disease which infects the upper air passages.

[Dr. Arkwright has prepared accounts of work done before the war, of the state of knowledge at the outbreak of war, of the bacteriological work done during the war, including serological observations, of cultural work, and of notable contributions to the pathology, and also a bibliography which it has not been found possible to reproduce here.]

Diagnosis and Classification of Meningococci.

Among the many points of importance in the great mass of work that has been done during the war the chief interest centres round the classification of the meningococcus, both for practical purposes and from the theoretical standpoint. On this largely depends the ability to recognize the meningococcus in the nasopharynx and the means to be used for this purpose, and also the desirability and possibility of detecting and isolating carriers.

The diagnosis and classification of the meningococcus may be made (1) by the morphology of the cocci and their colonies, and by cultural means, including fermentation; (2) by agglutination with a polyvalent serum with or without absorption of agglutinins, or (3) by agglutination and absorption of agglutinins, using monovalent serums.

It is agreed that meningococci can be divided by agglutination into two or more main groups, but the sharpness of definition of the principal groups and the number and distinctness of small accessory groups which contain so-called aberrant strains are not fully agreed upon.

The possibility of further division of the two chief groups is also generally accepted; but the number of subgroups and the degree to which they overlap, their constancy in culture and in the human body, and the need or desirability of these finer subdivisions for practical purposes, are matters about which there is wide divergence of opinion.

Gordon and his co-workers maintain that 98 per cent. of spinal meningococci fall readily into four groups which are quite distinct and have only minor bonds of relationship; that these four groups can be distinguished by simple agglutination, but that the grouping is assisted and confirmed by the method of absorption of agglutinins. On the other hand, Griffith and Scott appear to have quite clearly shown that the classification into four groups is not inclusive of all strains, and that it is artificial and only accomplished by an arbitrary selection of standard strains for making the type serums. Moreover, that classification on these lines might equally well be carried still further, and that each of the two main groups might be divided into three or four subgroups, and that the more it is attempted to establish these finer subdivisions, the more other points of relationship come into prominence and the less sharply defined the main two groups appear to be.

Griffith and Scott further support these observations on the spinal strains by showing that strains from the nasopharynx have a special tendency to show affinities with both of the main groups or two or more subgroups. They also hold that their researches show that strains from the nasopharynx can be diagnosed by cultural means alone, and that agglutination tests, if sufficiently comprehensive, support these cultural results.

The General Position of Serological Diagnosis.

Before considering further the diagnosis of the meningococcus by serological tests it will be well to glance briefly at the general question as it relates to other kinds of bacteria.

In studying any pathogenic organism one of the most important data to settle is how this micro-organism can be identified conclusively when met with, and how it can be distinguished from other similar micro-organisms. Closely connected with this problem is the question why the organism is considered to be pathogenic and the cause of the disease with which it is connected.

To take the latter point first, in spite of the formulation and to a large extent the acceptance of the famous postulates of Koch, nevertheless these desiderata are only forthcoming in a very few of the diseases which are believed to be due to definite micro-organisms. In other cases the thesis that the disease is produced by infection with a certain bacterium is generally supported by analogy and by a mass of circumstantial evidence giving a strong *a priori* foundation for the claim, which is finally clinched by some one or more very strong special arguments which are different in each individual disease. These "conclusive" arguments are of very different kind and value in different cases.

Thus the *B. typhosus* cannot readily be made to reproduce a disease like enteric fever in animals (unless Besredka's recent work on the sensitization of rabbits with bile is confirmed). Its acceptance, however, as the infective agent is amply justified by its almost constant presence in the blood of cases during the first week of fever, and the appearance of antibodies to the bacillus in the blood of the patient during the course of the disease. In addition to this there are very definite instances of laboratory infection. *B. dysenteriae* produces death in rabbits and lesions in the bowel very like those which are found in man, though in animals it does not as a rule produce a continued state of disease.

The meningococcus, while it is not capable of producing a characteristic infective disease in animals, is constantly found in the meninges and purulent cerebro-spinal fluid of a certain class of cases of meningitis, which can also be diagnosed clinically, and *post mortem* on macroscopical evidence.

In any case the definite and certain recognition of a pathogenic bacterium after its removal from the body is of great importance, otherwise the statement that an organism is the cause of the disease, since it is in constant association with the disease, merely becomes the equivalent of saying that some uncharacterized bacterium is constantly found under the circumstances—a very different statement.

B. typhosus, though not so susceptible to the animal test as some other pathogenic bacteria, possesses the characteristic of remarkable uniformity when isolated, and thus it has the advantage of being readily recognized. The agglutination of the *B. typhosus* by specific serums has not only been studied for a longer time and probably by more workers than that of any other kind of bacterium, but it has been the special typical instance which has been taken when the subject of bacterial agglutination has been investigated. It so happens that the reaction between the *B. typhosus* and its appropriate serum is more uniform than in most cases, if not more than in any other instance, and as the serological relationships of the *B. typhosus* to other bacteria are with few exceptions remote, they seldom, if ever, complicate the diagnosis of the bacillus.

Value of Agglutination.

These facts relating to the agglutination of the *B. typhosus*—namely, that it was the prototype of all bacterial agglutination, that it is singularly constant and highly specific—appear to me to have given a prestige to the agglutination test which it does not quite deserve.

Even amongst such constant organisms as *B. typhosus*, *B. paratyphosus* A, and *B. dysenteriae* (Shiga) it must be remembered that inagglutinable and spontaneously agglutinable strains or cultures occur and thus depreciate the value of the test. I have recently been able to demonstrate¹ that variations of the *B. dysenteriae* (Shiga) can readily be obtained which show very greatly altered agglutination reactions.

If the value of agglutination for the identification of almost any other kind of bacterium is considered it is found that the course is not such plain sailing, though some pathogenic organisms present a fair degree of uniformity—for example, *B. paratyphosus* A and *B. dysenteriae* (Shiga). On the other hand, the attempt to identify by agglutination strains of the *B. paratyphosus* B group or *B. dysenteriae* (Y-Flexner) may immediately raise difficulties, and the unity or multiplicity of the vibrios of cholera and paracholera are matters which are far from settled. In the case of the *B. paratyphosus* B group (as has recently been detailed by Schütze)² cultures of bacteria from swine and other animals and from food poisoning cases may agglutinate equally and with the same serums as *B. paratyphosus* B strains from cases of "enterica" in man; and some strains resembling *B. paratyphosus* B, which have also come from enterica patients (*B. paratyphosus* C or Hirschfeld strains), do not agglutinate with all true *B. paratyphosus* B serums, though closely resembling this bacillus culturally, and being agglutinable by serums made with other strains of the same bacillus.

The *B. dysenteriae* group also present difficulties of the same kind. An agglutination test with a serum prepared from a casually chosen strain of this group is of little value for diagnostic purposes, for its reaction embraces only a few members of the group. In this case, however, the diagnosis is much helped by the fact that at any rate a very large number of strains from cases of dysentery, selected in the first instance by their sugar reactions, are agglutinated by a serum prepared with one well known strain—the *B. dysenteriae* Y of His and Russell, as was shown by Morgan (1911).³

Absorption of Agglutinins.

In order to overcome the difficulties of these uncertain and apparently non-specific reactions, the method known as absorption of agglutinins, devised by Castellani for dealing with streptococci and used by him for the elucidation of mixed infections, was adopted by Boycott and Bainbridge for investigating the paratyphoid group.

Schütze (1920) has repeated and extended the work of Uhlenhuth and Bainbridge, and has shown that the

B. paratyphosus B—Aertrycke group may be divided by the absorption technique into at least nine subgroups, and a number of further subdivisions which he calls substrains. A "substrain" is separable from the "superstrain" with which it is connected by the result of an absorption experiment in which the "substrain" is shown to absorb only its own agglutinins from a serum made with the "superstrain." He also records the derivation of a "substrain" from its corresponding "superstrain" in culture.

It seems certain that an attempt to classify this group by simple agglutination alone is not quite satisfactory, and that if absorption of agglutinins is taken as the criterion, the multiplicity of groups and subdivisions becomes confused and excessive for practical purposes. The researches of Andrewes and Inman (1919)⁴ on the grouping of *B. dysenteriae* (Flexner-Y) by agglutination and absorption of agglutinins have focussed attention on the very numerous varieties of this group, also as regards their serological properties.

This inability to rely on the serum prepared from one strain for the identification of others, and the facility with which groups apparently uniform may be divided by "absorption" into a large and indefinite number of subgroups, are therefore phenomena not confined to any one group of bacteria. The widespread character of this experience seems to throw light on the limits of these methods of classification.

From these considerations it may be concluded that agglutination amongst nearly allied bacteria is an extremely valuable diagnostic test, but only final and decisive in a rather limited number of cases, and even then not infallible. It has the advantage of being a fairly inclusive test if due precautions are taken to select a suitable (polygerent) strain with which to prepare the serum.

Absorption of agglutinins for purposes of classification has the disadvantage that by its means strains which appear to be closely allied on other grounds are separated, and cross-connexions are found which make the formation of definite groups difficult and their limits uncertain. One undoubted merit of "absorption" is that it can be used when direct agglutination fails because the culture under examination is (1) inagglutinable, though capable of absorbing, or (2) spontaneously agglutinable, or (3) when the serum available is polyvalent and has far too large a scope for the purpose in hand.

If it were certain that an exact knowledge of the agglutinable substance, agglutinogens and agglutinins, were of unique importance for establishing the true relationship by descent of bacteria, then the method of absorption of agglutinins would have much to recommend it for classificational purposes. It is at the best, however, more complicated than simple agglutination and therefore more liable to certain errors. The assumption of its prime importance cannot at present be considered as fully justified in the face of the unexpected divisions and cross-relation-

ships revealed by this technique, and the very striking changes which some strains undergo in culture.

If, however, it were maintained that a sound classification of pathogenic bacteria would be more securely based on the reactions produced in the animal body than on their assumed evolutionary kinship, it would be necessary to consider whether agglutination and absorption had any special relation to infectivity, virulence, or toxin production. These two classes of characters have, however, been found to vary independently in some cases, as Tulloch (viii, 1919)⁵ has shown for the agglutination types of *B. tetani* and their toxin, and Miss Robertson⁶ for the *Vibrio septique*.

Certain variations in *B. dysenteriae* (Shiga) which I have found to occur *in vitro* were associated with remarkable changes in the agglutination properties, but did not seem to be closely related to the toxicity of cultures. In the same way the cases of cerebro spinal fever associated with different serological types of the meningococcus have not been shown to have any considerable differences clinically.

The above remarks are not intended as a criticism of the very arduous work undertaken as a research into the uses and limits of the method of absorption of agglutinins, and as an attempt to determine how far relationship (evolutionary or physiological) can be detected by this means. It is only when it is maintained that this method is final and necessary as a means of elucidating relationship and establishing a natural classification that the claim appears to outrun the evidence.

Absorption of Agglutinins in the Meningococcus Group.

The test of absorption of homologous agglutinins from a serum was used by Gordon in order to diagnose more accurately meningococcus-like cultures, and he was led to exclude a number of strains which were found in the nasopharynx by this means alone. He was also enabled to place more definitely certain strains of Groups III and IV. Tulloch by this method detected three subgroups in Group II, and thus explained defects in the behaviour of this group.

Gordon's four groups never quite covered all the spinal strains, though he found that 98 per cent. could be classified. He and his colleagues found that, in order to be able to assign so large a proportion of strains to their appropriate groups, the technique of agglutination and absorption must be very exact as regards the details of the procedure, and that the time spent in immunizing the rabbits was of considerable importance; moreover, that the laboratory worker must be highly trained and in constant practice. Thus the work of others who found difficulty in reducing the strains to four distinct groups is criticized on account of the length of time over which the rabbits affording type serums were subjected to inoculations, since it was held that a prolonged immunization made the resulting serum too inclusive in its qualities.

The demand for such precision and hardly-come-by niceties of method requiring such arduous training unfortunately discounts as valueless much of the control work done by others. It recalls the technique by which Procrustes applied an arbitrary and inelastic standard to his guests.

Some of those who have experience of the cross-relationships demonstrable between different groups of meningococci, as evidenced by the occasional change-over of a strain from one group to another, the equal action of serum prepared from one strain on cocci from different groups, or the equal agglutination of one strain by serums made from cultures belonging to different groups, will hesitate before adopting a procedure which leads to further subdivisions.

The knowledge that the allied class of gonococci also can readily be divided into subgroups which independently cross-agglutinate with meningococcus groups, and that some strains of gonococci more nearly resemble serologically some meningococci than other groups of gonococci, makes one pause before accepting such methods of classification as final.

If it were proved that a knowledge of the subgroups were of value for the production of therapeutic serum or for any such specific purpose, then this classification would become immediately of great importance.

Isolation of Carriers.

Attempts to correlate the isolation of carriers with the prevention of the disease are very difficult, and, considering the apparent capriciousness of the distribution of the disease, and the comparative rarity with which it attacks more than one person in a house or more than one soldier in a hut, and the large number of carriers often found, the chances of demonstrating the success of an isolation policy must be very rare.

However, a considerable advance has been made by accumulated knowledge of the rise and fall of cases in a small area with the change in the carrier rate. The practical measures required to obviate a serious outbreak are matters which have been especially studied by Dr. Glover (1918).⁷ In my opinion the position of the carrier question may be stated as follows:

1. The question whether carriers should be isolated depends, in the first instance, on our ability to recognize the meningococcus certainly in the nasopharynx. It must be borne in mind that at present no really final answer can be given to the question whether a given Gram-negative coccus is truly an infective meningococcus or not, unless it comes from the meninges.

2. For diagnosis, cultural tests if very carefully carried out are probably at least as good as any other, but in view of the large number of somewhat similar organisms in the throat apart from meningococci, especially if regard is had to certain epidemics of bronchitis, etc., it seems desirable to use an agglutination test in addition. Polyvalent horse serum is probably too inclusive, and single rabbit serums would require to be too numerous. A polyvalent rabbit serum prepared with

several strains from the meninges of as different serological reactions as possible appears to be the most suitable diagnostic serum.

3. The policy of isolation, even if desirable on other grounds, must depend on the proportion of the population found to be carriers. If this proportion is large—for example, 10 or even 50 per cent. in non-contacts—the procedure seems to be an impossible one.

4. It seems doubtful if we know much about the proportion of carriers in the population between and at the earliest stages of epidemics, and the relation of the rise and fall of the numbers of cases to those of carriers at different periods of the epidemic. To ascertain these facts with any accuracy it seems almost essential that the same workers, using the same technique, should make observations at intervals over very long periods.

5. In all but very exceptional circumstances the detection and isolation of carriers appears to be a proceeding which should not be attempted in the present state of our knowledge.

6. Various methods for the disinfection of carriers have been devised, but this most difficult problem has not met with any decisive solution.

Therapeutic Serum.

The best methods of preparing therapeutic serum, and whether it should be polyvalent or monovalent, are still vexed questions. They are very difficult problems, since there is no recognized laboratory test of the efficacy of the serum which can be taken as an indication of its clinical value. Virulence and toxicity tests of living and dead cultures, and of preparations of "endotoxin," have been tried on mice and guinea-pigs in this country, on the Continent, and in America, but as far as I can ascertain there is no agreement in different laboratories as to the value of these tests.

Gordon in January, 1918, published a method for preparing "endotoxin" and standardizing serum by testing its efficacy in protecting mice against the toxin. Dopter reported very good results with univalent serum. Flexner's serum made at the Rockefeller Institute, with which some of the best results were claimed, and which has been credited with good results in a relatively small number of cases during the recent epidemic, is polyvalent, and care is now taken to include both the meningococcus and the parameningococcus in the cultures used.

I should like to enter a plea for multivalent serum. Most of the best results recorded have been obtained with multivalent serums. The type of meningococcus in any given case cannot as a rule be known for a day or two, and the difficulty of having two or more serums available is great, and must much increase the practical difficulties and cost of treatment.

CONCLUSIONS.

To sum up what appears to me to be the present state of our knowledge as the result of the advances made during the war:

1. The position of the meningococcus as the causal organism has been confirmed. Cultural methods have

been improved and are at least as efficient as serological methods for diagnosis, but both means should be used.

2. The occurrence of two main groups of meningococcus corresponding to Dopter's meningococcus and parameningococcus has been recorded in all epidemic centres, and in about equal numbers.

3. These two main groups are capable of division into a further indefinite number of subgroups by making serums from a number of single strains for agglutination, and by the use of the absorption of agglutinins technique. Gordon's Types III and IV have a special prominence among these subgroups, but whether this is due to the specially selected strains for the diagnostic serums or to a greater definiteness of these two subgroups is not quite clear.

4. The meningococci from the nasopharynx compose a very ill-defined group, and though many agree absolutely with the commoner strains from the meninges, others correspond only to the rarer meningeal strains. Other strains, again, show no relationship to strains from the meninges by agglutination tests, but a close affinity is apparently revealed when they are used to make agglutinating serums. In these circumstances, and since no absolute criterion of a meningococcus is known, it is arbitrary either to include these among the meningococci or to exclude them.

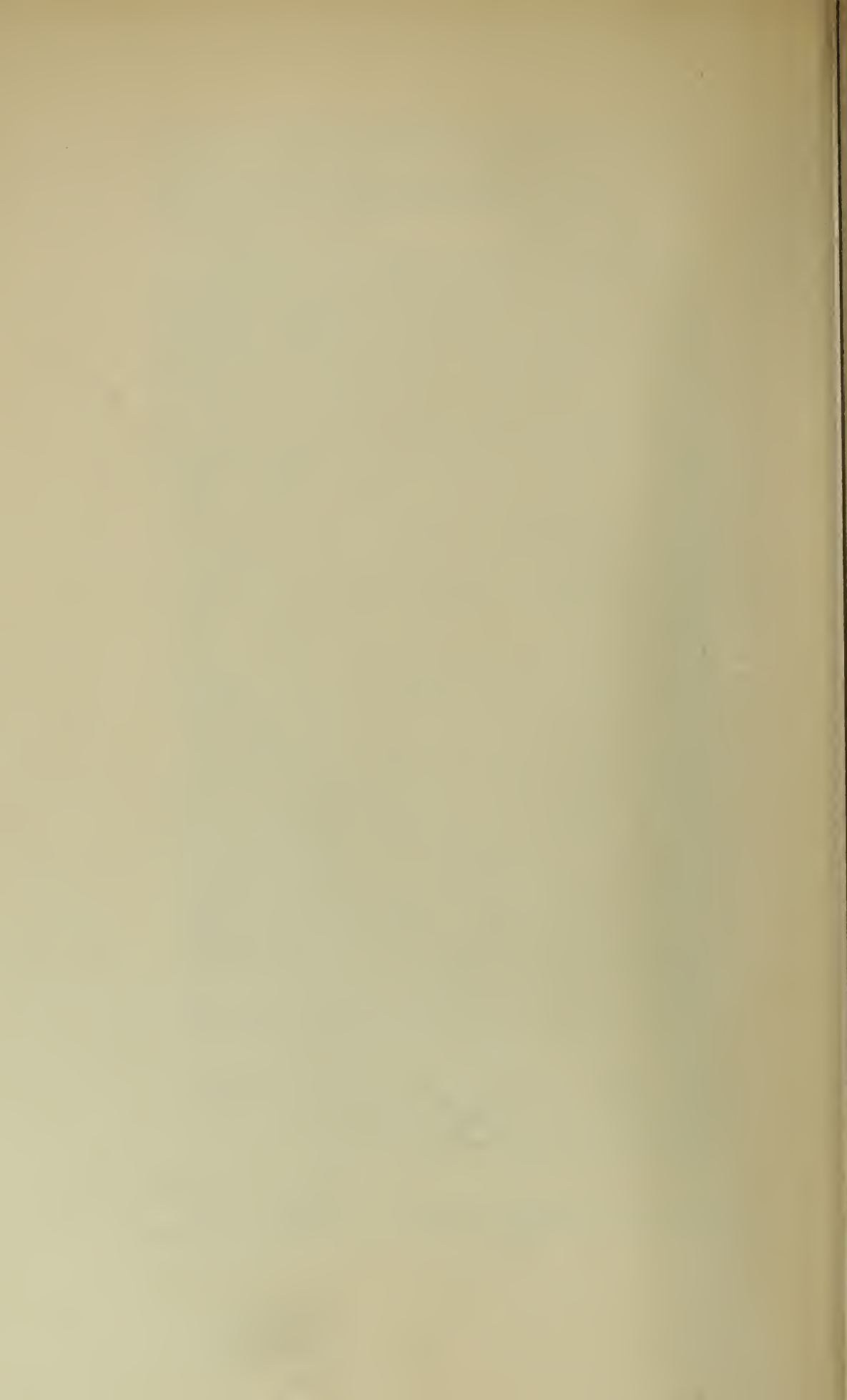
5. The contacts and "non-contacts" who carry the meningococcus in the nasopharynx during an epidemic are so numerous that it is neither necessary nor desirable to attempt isolation of carriers. It is very probable that even when cases of meningitis first appear at the beginning of an epidemic the number of carriers is often too high to make an isolation policy possible, but the data for forming an opinion on this point are not very numerous.

6. The manufacture of therapeutic serum still needs much research and observation. In order to gain more knowledge on the value of different methods of immunization of horses and of laboratory tests of the therapeutic efficiency of serum, it is very necessary that the reference numbers on phials of serum should be carefully noted by the medical men who administer the serum, and that the result of its use should be reported in all cases to those responsible for its manufacture.

Only in this way can evidence be obtained of the value of special methods of manufacture, and progress be made.

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³ *Journ. of Hyg.*, xi, p. 1. ⁴ Medical Research Committee, Special Reports, Series 42. ⁵ *Journ. of Hyg.*, xviii, p. 103. ⁶ *Journ. of Path. and Bact.*, xxiii, p. 153. ⁷ *Journ. of Hyg.*, xvii, p. 350.



Variation in Bacteria in Relation to Agglutination both by Salts and by Specific Serum

BY

J. A. ARKWRIGHT, M.D.

From the Bacteriological Department, Lister Institute, London, S.W.

VARIATION IN BACTERIA IN RELATION TO AGGLUTINATION BOTH BY SALTS AND BY SPECIFIC SERUM.¹

By J. A. ARKWRIGHT, M.D.

From the Bacteriological Department, Lister Institute, London, S.W.

(PLATE II.)

THE varieties of bacteria with which the present paper is mainly concerned belong to the category commonly called "spontaneously agglutinating" strains. They have been obtained from cultures of *B. typhosus*, *B. paratyphosus* B., *B. enteritidis*, and *B. dysenteriae*. Most of the observations here recorded have been made on Shiga's bacillus, but the writer has been working with similar variants of *B. typhosus* and *B. enteritidis* since 1912, and has already recorded some observations on a spontaneously agglutinable strain of *B. typhosus* (Arkwright, 1914¹).

TWO FORMS OF VARIANT.

The chief variants now to be described are two in number, which for reasons given subsequently have been designated the "S" form, which makes good stable emulsions in 0.85 per cent. solution of sodium chloride, and the "R" form, which agglutinates spontaneously in salt solution of this concentration.

Both these forms differ in some degree from the original parent cultures which may be regarded as the normal. The relation of the two variants to the normal is such that the latter appears to contain representatives of them both, or to be composed wholly or in part of the characters of the two forms which can, as it were, be split off from the normal. The normal, at any rate superficially, resembles more nearly the "S" form. The observations which show the "S" form to be distinctly different from the normal and those which concern agglutination by specific serum have reference almost entirely to *B. dysenteriae* (Shiga). These variants have proved constant in their characters when subcultured in ordinary nutrient broth at intervals of about one week and cannot therefore be classed as mere modifications due to the environment. The expressions "Variant" and "Variation"

¹ Received June 12, 1920.

are here used in respect of any change which is not merely due to temporary external conditions. What kind of variation is manifested by the forms "S" and "R" will be discussed later.

Cultures examined for "S" and "R" forms.

Micro-organism.	Ref. No.	Source.	Forms obtained.
<i>B. dysenteriae</i> (Shiga)	47	Capt. Gilmour, Italy.	Agar, 3 mths.
	190	" "	" "
	236	" "	" "
	177	" "	" "
	550	" "	" "
	862	" "	" "
	"L.I.P.M."	Lister Institute.	" "
	"Parker"	" "	" "
<i>B. dysenteriae</i> (Flexner-Y)	"Wynne"	" "	" "
	215	Capt. Gilmour, Italy.	" "
	573	" "	" "
	577	" "	" "
<i>B. typhosus</i>	"Simpson"	Lister Institute.	" "
	"Guy"	" "	" "
	"Lincoln"	" "	" "
	"F"	Dr J. C. G. Ledingham,	" "
<i>B. paratyphosus</i> B.	"Schottmüller"	Lister Institute.	" "
	"Tidy"	" "	" "
<i>B. enteritidis</i> (Gaertner)	"Van Ermenghem"	" "	" "
	"Lab"	" "	" "
	"Rat"	" "	" "

The strains of *B. dysenteriae* obtained from Captain Walter Gilmour, R.A.M.C., were isolated by him in Italy in 1918, and very kindly sent to me in the autumn of that year. They were examined by me in December 1919 and subsequently.

The strain of *B. typhosus*, "F," was isolated by Dr Ledingham from a carrier. The "R" form was obtained by him by plating on MacConkey's medium after growth in immune typhoid rabbit serum.

All the original cultures mentioned above and the "S" and "R" forms derived from them gave the typical cultural reactions in carbohydrate media and in milk; they also behaved normally as regards the production of indol and motility, except that the "R" forms of bacteria which were normally motile showed little or no motility.

Both the "S" and "R" forms and the sub-variant "RV" of *B. dysenteriae* (Shiga) 550 were fatal to rabbits when injected subcutaneously or intravenously in very small doses, but no attempt was made to find the M.L.D.

CHARACTERS OF THE VARIANTS.

The most obvious distinguishing characteristic of the "R" form is the property which emulsions possess of forming clumps and precipitating in solutions of sodium chloride. The percentage of salt which leads to agglutination in the case of different strains varies considerably. Often emulsions of the "R" form are stable in saline solution of half or one-quarter the usual strength (*i.e.*, in solutions containing 0.42 or 0.21 per cent. NaCl); but sometimes clumping and precipitation occur in saline diluted eight times (0.1 per cent.), or even in weaker solutions. Agglutination occurs in much weaker solutions of calcium chloride (Table I.) as is usually the case with

emulsions or colloidal solutions which are precipitable by weak electrolytes (*e.g.*, Tulloch, 1914³⁵).

In liquid culture media the "R" form is distinguished from the "S" form and from the normal by the appearance of the growth. Cultures of the "R" form in ordinary nutrient broth or peptone water produce a deposit at the bottom leaving the liquid above clear, instead of making it turbid, as do cultures of the normal or the "S" form. This precipitation of the bacteria in the culture tube is due to the salt content of the medium, for if the broth is diluted with distilled water to one-half or one-quarter of its original strength, or if the medium is made with less salt, the growth of the "R" form also causes uniform turbidity with little or no deposit.

On solid media the differences between the colonies of the two forms are often very characteristic. (Plate II., Figs. 1, 2, 3, and 4.)

The main characters given below apply to all the kinds of bacteria mentioned, though there are individual differences and the descriptions given are not intended to be exhaustive. The appearances to be described refer to discrete colonies on plates of ordinary nutrient agar which have been at 37° C. for about twenty-four hours. Similar appearances are seen on plates of MacConkey's medium, and the peculiarities of the "R" colonies may then be rather exaggerated.

Colonies of the "S" form have a smooth and glistening surface, are raised, dome-shaped, and round, with smooth, regular, well-defined margins: they are translucent, and when examined with a low power of the microscope (1 inch) appear quite smooth or only very finely granular.

The shape of colonies of the "R" form is more varied, but their appearance is often quite distinctive. In the most characteristic form they are larger than the "S" colonies, flat and thin, with a slight central boss and have a jagged or irregular, waved or indented margin; the surface is coarsely granular, recalling the appearance of very finely-grained morocco leather, and may be marked by irregular branching lines. By transmitted light they appear slightly opaque, or have a frosted appearance, especially when seen through a hand lens: they are seen to be coarsely granular under a low power of the microscope. The irregularity of the surface has led to this variant being called the Rough ("R") form in distinction from the Smooth ("S") form. The irregular shape of the "R" colonies is probably due to the way in which the individual bacilli cohere in the presence of sodium chloride, forming many centres of growth instead of spreading evenly from a single centre.

The variety of colonies met with is so great and the differences between them often so indefinite that attention has purposely been directed only to the more obvious characters which are definitely associated with especial behaviour in broth cultures and in emulsions in salt solution. It is not, however, intended to convey the impression that colonies of the "S" and "R" types can always be readily distinguished. The behaviour on agar and in broth must be examined before a variant is definitely regarded as of the "S" or "R" form.

Some varieties of colonies have been met with which corresponded

with those described by Baerthlein (1912,³ 1918⁴), but they appeared for the most part to be intermediate between the "S" and "R" forms and to yield uncertain results when tested in respect to their agglutinability by salt.

Besides the colonies which appear to be definitely "S" or "R" in character, others of a mixed type are often seen which at first are smooth and later become irregular in outline and more or less uneven on the surface. Colonies in part translucent and in part opaque or granular are also seen which appear to be composed of bacilli of both "S" and "R" form. These latter colonies look very much like mixed colonies of two entirely different kinds of bacteria, whereas they are really composed of the two forms imperfectly blended.

Different colonies of the "R" form of the same kind of bacillus may be very unlike each other in appearance. (See Plate II., Fig. 4.)

When the "R" form is replated, marked differences often appear in the colonies which seem to indicate degrees of specialisation of this variety, *e.g.*, some colonies may definitely be larger, thinner, and much rougher on the surface, and this difference is apparently associated with a tendency to agglutinate and precipitate in weaker salt solution.

In the case of *B. dysenteriae* (Shiga) I have found one sort of colony commonly associated with the characters of the "S" form and two with the "R" form. The "S" colonies are round, shiny, smooth, sharply defined, raised, domed, and translucent; the "R" colonies may be (1) flat and thin with very irregular outlines and rough, granular, or wrinkled surface; they resemble the "R" colonies of *B. typhosus*. These are larger than the "S" colonies and may be 4 to 6 mm. in diameter; (2) more commonly they are raised and moderately thick, but flattened, rather larger than the "S" form, and have indented or slightly jagged margins; and are also somewhat opaque. They are seen to be granular under a low power of the microscope, and may appear finely granular or frosted with a hand lens; the surface is usually dull. These latter colonies are associated with a sliminess which is apparent in emulsions.

One other type of colony, "RV" (= "R" variant), must be described, however, since it was definitely associated with the property of forming clear cultures with a deposit in broth and of agglutinating spontaneously in salt solution. These colonies were very small, round, smooth, and of an unusually coherent or sticky consistency. They appeared on agar plates inoculated from a culture of *B. dysenteriae* (Shiga) 550 form "R," which had been growing in pure horse-serum at 37° C. for seven days. Only this type of colony was present on the plates inoculated on three different occasions, *i.e.*, the seventh, fourteenth, and twenty-first days of growth in horse-serum. These colonies were difficult to subculture, since when picked off to broth they almost invariably failed

to grow. Subcultures were, however, obtained on agar or in glucose peptone water, but these at first died after two or three days. It was not till after several subcultures on agar at short intervals that growth could be obtained in broth or on agar which was viable after seven days. The resulting free growth on agar formed emulsions in distilled water which were slimy and agglutinated in 0.1 per cent. sodium chloride solution. The results of agglutination experiments with specific sera prepared with this variant are recorded later in this paper.

CHANGES IN OTHER CULTURAL CHARACTERS AND MORPHOLOGY ASSOCIATED WITH AGGLUTINABILITY IN SALT SOLUTION.

(1) A surface film in broth cultures is very often formed by the "R" form whether of *B. typhosus* or *B. dysenteriae*, but the "S" form occasionally also forms a film.

(2) Sliminess of emulsions from agar slopes is very often present in the "R" form of Shiga strains, especially in cultures from flat, thick "R" colonies, and has also been noticed to a smaller degree in the "R" form of *B. dysenteriae* (Flexner-Y), and of *B. typhosus*. It has not always been noticeable in my Shiga "R" forms, and certainly may be absent in the "R" forms of *B. typhosus* and *paratyphosus*. This character appeared to be associated with large swollen forms of the bacilli, but capsules could not be demonstrated and no large swollen mucoid colonies appeared on agar as in the case of the "mucoids" described by Revis (1910³⁰) and Fletcher (1918¹⁴).

(3) The morphology of the bacteria varied very much in different one-day old agar cultures, and to a large extent varied independently of the "S" and "R" characters. The individuals of the "R" type were usually rather wider and had rounded ends. Occasionally the papillated or shagreened surface of rough colonies was associated with very long thread-like bacteria.

When the slimy character was well marked in the "R" form, some of the bacteria were usually very much swollen, *e.g.*, two or three times the diameter of normal individuals and were often very irregular in shape. Sometimes they were forked or had knob-like protrusions on their side, and many ill-stained forms varying very greatly in size and shape were also seen. In these cultures there were also very great differences in the size and shape of the well-stained bacilli, both as regards breadth, length, and the uniformity in width of single bacilli, and these irregularities were often not confined to the "swollen" individuals. (See Plate II., Figs. 5 and 6.)

Source and Frequency of Occurrence.—The source of the "R" variants has been almost invariably old broth or agar cultures. They may be obtained from most strains if a culture which has been kept at room temperature or in the incubator for a month or more is plated out, but

the proportion of such colonies varies very much in different strains, and some strains have not so far been found to yield the "R" form. Of nine agar cultures of *B. dysenteriae* (Shiga) over a month old all yielded this variety; eight did so at once when plated, and the other strain (Wynne) which did not on the first plating, did so on a subsequent occasion.

Degree to which "R" Characters are manifested by Different Cultures.—The agglutinability by salt is to a certain extent a matter of degree. Most "R" variants obtained from *B. typhosus*, *B. paratyphosus* B., and *B. enteritidis* (Gaertner) were agglutinated in 0.85 per cent. but not in 0.42 per cent. NaCl. Variants of *B. dysenteriae* (Shiga) were usually not quite stable in 0.42 per cent., but did not agglutinate in 0.21 per cent. NaCl; again a variant obtained from *B. typhosus* (Guy), one from *B. dysenteriae* (Flexner-Y) 573, and the variant "RV" obtained from *B. dysenteriae* (Shiga) 550 were stable in 0.05 per cent. but not in 0.1 per cent. (*i.e.*, in 1/16 but not 1/8 saline). (Table I.)

TABLE I.—*Agglutination of "S" and "R" Forms of Bacteria by Salts.*

	NaCl.					CaCl ₂ .									D. W.
	$\frac{M}{1}$	$\frac{M}{2}$	$\frac{M}{4}$	$\frac{M}{8}$	$\frac{M}{16}$	$\frac{M}{2}$	$\frac{M}{4}$	$\frac{M}{8}$	$\frac{M}{16}$	$\frac{M}{32}$	$\frac{M}{64}$	$\frac{M}{128}$	$\frac{M}{256}$		
<i>B. typhosus</i> Strain "Guy"															
"S" form .	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
"R" form .	+++	+++	+++	++	-	++	+++	+++	+++	+++	+++	+++	+++	+++	-
<i>B. enteritidis</i> (Gaertner)															
"S" form .	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
"R" form .	-	-	++	+	-		+++	+++	+++	+++	+++	+++	+++	+	-

$\frac{M}{1}$, $\frac{M}{2}$, $\frac{M}{4}$, etc. = Molar $\frac{1}{1}$, $\frac{1}{2}$, $\frac{1}{4}$, etc.

+++ , ++ , + = complete, well marked, and slight agglutination.

NaCl $\frac{M}{1}$ = 5.8%. CaCl₂ $\frac{M}{2}$ = 5.5%. D.W. = Distilled Water.

Reversion.—When after selection of the colonies one of the variants "S" or "R" has been obtained apparently quite pure, a change into the other form is not so easy to bring about as when unselected cultures have shown the "S" or "R" characters. The reason of this would appear to be that in the latter case the character of the culture is not "fixed," *i.e.*, the culture does not consist of uniform members. Thus *B. typhosus* (Guy) was separated into two forms "S" and "R" by plating twice from an old culture. When subcultured weekly the two forms have remained distinct, but when subcultured daily the "R" form showed some turbidity at the 4th subculture, and at the 10th subculture showed much turbidity and hardly any deposit, in this way indicating that the culture contained the "S" form in a pure or nearly pure state. A culture of the "R" form of *B. paratyphosus* B. (Tidy) became slightly turbid at the 2nd or 3rd daily subculture in broth,

and at the 18th subculture the turbidity was uniform and the deposit only very slight. The estimation of this change by the characters of broth cultures alone is not always quite easy, because the "R" variants often form a surface film in broth, and this readily breaks up and causes slight temporary turbidity. The presence of a film is, however, shown by the clearing of the broth and the appearance of a deposit on the following day. The changes found in broth cultures need to be corroborated by plating out and observing the shape and appearances of the colonies. When a culture is changing from "R" to "S" or from "S" to "R," but perhaps less so in the latter case, the colonies are often of a mixed or indefinite character till a late stage in the change, when pure "S" or "R" forms of colony can be picked off plates.

B. dysenteriae (Shiga) 550 form "R" when subcultured daily showed no sign of changing into the "S" form either by production of turbidity in broth or by the appearance of "S" colonies on plates when subcultured daily forty-eight times, but the 49th, 50th, 51st, 52nd, and 53rd subcultures were slightly turbid and some smooth or partly smooth colonies appeared on plating. These, however, did not behave towards salt solution and specific serum like the "S" form though culturally like the original stock culture.

B. dysenteriae (Shiga) 862 "R" showed slight turbidity in broth at the 18th, 19th, 20th, and 21st subcultures, but no distinctly smooth colony could be found after plating.

Similar results were obtained with other strains of *B. dysenteriae* (Shiga). On the other hand, a broth culture of "Shiga" 550 "S" at the 25th daily subculture was plated when a month old and still gave all smooth colonies; the 40th subculture when plated after nineteen days at 37° C. gave a few rather opaque colonies which in broth formed a large deposit with slight turbidity, and on replating this broth some definitely "R" colonies appeared which were semi-opaque and irregular in outline. These observations may be summarised by saying that the reversion from pure variants of *B. dysenteriae* (Shiga) is slow, and the first colonies of changing type are apt not to be pure. The mixed type of colony is more often seen when a culture is changing from "R" to "S" than when the reverse change is proceeding. The "R" form can almost always be obtained from the "S" form, but the "S" form cannot so uniformly be obtained from "R" cultures especially in the case of *B. dysenteriae*.

Changes observed as regards Specific Agglutination.

Method of Agglutination.—The macroscopic method was used with the assistance of microscopical examination of the deposit in some cases. The emulsions were always made in distilled water and the serum was diluted with physiological saline (0.85 per cent. NaCl), or a weaker solution if the emulsions were very readily agglutinated by salt. The emulsions and dilutions of serum all contained 0.5 per cent. phenol.

The agglutination was carried out in a 37° C. incubator for four hours or sometimes longer. Agglutination was considered to be complete if the liquid was quite clear and if there was a well-defined deposit distributed over the concavity at the bottom of the test-tube.

Examination of the "Shiga" strains was first made by means of a stock agglutinating serum which had been prepared from three strains of Shiga's bacillus and had a titre for stock strains of 1/600 to 1/800.

In order to test the agglutination of the "R" form, it was necessary to use 0·2 per cent. NaCl solution, since emulsions in stronger saline were not always stable. This strength was therefore used for all strains and forms of *B. dysenteriae* (Shiga). (Table II.)

TABLE II.—*Agglutination of B. typhosus Emulsions of Normal, "S" and "R" Forms, with Specific Serum $\frac{1}{900}$ and Varying Salt Content.*

NaCl.	$\frac{M}{6}$ 0·97%	$\frac{M}{12}$ 0·48%	$\frac{M}{24}$ 0·24%	$\frac{M}{48}$ 0·12%	$\frac{M}{96}$ 0·06%	$\frac{M}{192}$ 0·03%	D.W.
<i>B. typhosus</i> , Normal .	+	+++	+++	+++	++	+	-
" S " .	++	+++	+++	+++	+++	++	-
" R " .	-	+++	+++	+++	+++	+	-

It is well known that the strength of salt required to produce agglutination of sensitised bacteria is much less than that commonly used. With a moderately strong dilution of serum the optimum concentration of salt is sometimes less than 0·85 per cent. and a concentration of about 1/25 of the ordinary strength (*i.e.*, 0·034 per cent. NaCl) is quite sufficient. It has, however, been shown that when the serum is highly diluted, rather more salt is required than when the serum is stronger.

Type of Agglutination: Size of Clumps.—It has often been noticed that the type of agglutination is not the same for all kinds of bacteria nor even for different strains of the same kind. For instance, the agglutination of some strains of *B. typhosus* (*e.g.*, "Simpson," *vide* p. 37) takes place in large loose clumps which settle near the bottom of the tube in a loosely flocculent mass like a "cumulus," whilst other strains such as "Guy" settle in a dense concave layer and the individual clumps are smaller. Dysentery bacilli as a rule agglutinate in small dense clumps and form a thin granular layer distributed over the concave surface at the bottom of the tube, but the size of the individual clumps may vary very much. In some strains, on shaking the tube, the deposit breaks up into large pieces which are with difficulty shaken into a finely granular emulsion; in other strains, the deposit shakes up more readily and a few large clumps are then seen, but the resulting emulsion consists chiefly of small clumps. In still other strains (especially perhaps of *B. dysenteriae*, Shiga) the deposit when shaken up forms a

fine muddy emulsion in which no clumps can be seen with the naked eye, or even a hand lens ($2\frac{1}{2}$ -inch focus); at least this has been the experience of the writer during routine examination of the fæces of dysentery patients. A drop of this resulting emulsion put under the microscope is seen to consist of a suspension of small dense clumps containing four to twenty bacilli each. When an emulsion of the "S" form of *B. dysenteriae* (Shiga) is agglutinated the deposit is dense, and when shaken gives a suspension of large clumps. The deposit formed by agglutinating the "R" form, however, when shaken up forms an emulsion appearing uniformly turbid, the clumps being too small for recognition until a low power ($1/6$ inch) of the microscope is used. Even in the case of "R" emulsions, however, the appearance of the agglutinated deposit is quite characteristic of true agglutination as regards its distribution over the concave bottom of the tube, and as distinguished from the deposit in control tubes in which the unagglutinated bacteria are collected in a very small compass at the centre of the concavity. These differences between the agglutination of "S" and "R" forms occur whether the agglutination has taken place at 37° or at 55° C. When the precaution is taken of using weak salt solution (0.2 per cent. NaCl), and the different types of agglutination by the two forms are taken into account, it is found that both forms "S" and "R" of *B. dysenteriae* (Shiga) 550 agglutinate to approximately the same titre, $1/640$, as the parent strain with a stock serum, prepared from three strains of Shiga.

Absorption of Stock Serum.—These experiments were carried out by mixing 1 c.c. of serum diluted $1/10$ in 0.85 per cent. salt solution with 1 c.c. of a strong emulsion of bacilli in distilled water; the emulsion was made by adding the growth on one agar slope to 1 c.c. of water. After incubation at 37° for two hours the mixtures were put in the cold room till the next day and then centrifuged. The mixtures contained 0.5 per cent. of phenol. (Tables III., IV.)

TABLE III.—*Agglutination by Stock Specific Serum, before and after Absorption by B. dysenteriae (Shiga), Strain 550, Forms "S" and "R." The Serum diluted with Solution of NaCl 0.42 per cent.*

Stock Serum v. <i>B. dysenteriae</i> (Shiga).	$\frac{1}{200}$	$\frac{1}{400}$	$\frac{1}{800}$				NaCl. 0.42%
<i>Unabsorbed</i> —							
Emulsion 550 "S" .	+++	+++	+				—
„ 550 "R" .	++	++	+				—
<i>Absorbed by 550 "S"</i> .	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$	$\frac{1}{1280}$	
Emulsion 550 "S" .	+++	—	—	—			—
„ 550 "R" .	+++	+++	+++	++			—
<i>Absorbed by 550 "R"</i> .							
Emulsion 550 "S" .	+++	+++	+++	+++	++	+	—
„ 550 "R" .	+++	++	+	—	—	—	—

TABLE IV.—*Agglutination of "S" and "R" Forms of Heterologous Strains of B. dysenteriae (Shiga) by Stock Specific Serum before and after Absorption with B. dysenteriae (Shiga), Strain 550, "S" and "R" Forms. Diluent 0.21 per cent. NaCl.*

Stock Serum.	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$	NaCl. 0.21%
<i>Unabsorbed—</i>					
<i>B. dys. 47 III. "S"</i> .	+++	+++	+++	+++	—
<i>47 v. "R"</i> .	+++	+++	++	+	—
<i>177 II. "S"</i> .	+++	+++	+++	++	—
<i>177 IV. "R"</i> .	+++	+++	+++	+	—
<i>Absorbed by 550 I. "S"—</i>					
<i>B. dys. 47 III. "S"</i> .	+++	—	—	—	—
<i>47 v. "R"</i> .	+++	+++	+++	++	—
<i>177 II. "S"</i> .	+++	—	—	—	—
<i>177 IV. "R"</i> .	+++	+++	+++	+	—
<i>Absorbed by 550 II. "R"—</i>					
<i>B. dys. 47 III. "S"</i> .	+++	+++	+++	+++	—
<i>47 v. "R"</i> .	+	+	—	—	—
<i>177 II. "S"</i> .	+++	+++	+++	+++	—
<i>177 IV. "R"</i> .	++	—	—	—	—

The results of the absorption experiments showed that serum absorbed with the "S" form of Shiga 550 had lost the greater part of its power of agglutinating form "S," whether this was derived from strains 550, 47, or 177, but that it still agglutinated the "R" form derived from these strains nearly as well as before. After absorption with form "R" derived from 550 this form, whether derived from 550, 47, or 177 was subsequently very feebly agglutinated, whereas form "S" from all these three strains was agglutinated as well as before absorption of the serum.

It was also found that absorption with the "R" form of 550 much diminished the agglutinating action on form "R" of strain 236, but absorption with the "S" form of 550 did not do so; and conversely the "R" form of 236 absorbed the agglutinins for the "R" form of 550 but not for the "S" form of 550.

The "S" form of strain 236 of Shiga has not so far been examined.

Agglutination with Special Single Sera.—Single sera were prepared from rabbits by inoculating intravenously emulsions of "S" and "R" forms of *B. dysenteriae* (Shiga) which had been killed by heat. The exceptional form "RV" was also inoculated into a rabbit. All three forms were found to be very fatal to rabbits, but after two or three inoculations of very small doses sufficiently good agglutinating sera were obtained.

The "S" serum obtained by two inoculations agglutinated 550 "S" up to a titre of about 1/320 but 550 "R" scarcely above 1/80, whereas the "R" serum agglutinated 550 "R" in a dilution of 1/320, but did not affect 550 "S" above 1/80. Six other strains of Shiga (*Parker, L.I.P.M.*, 190, 47, 177 and *Wynne*) which had also been split into "S" and "R" forms were also tested with both sera "S" and "R."

direct agglutination and by absorption, and there was only slight cross-agglutination. The "S" serum was of much higher titre (1/5120) than the "R" serum (1/1280).

It was found necessary to use 0.1 per cent. NaCl for this test, since the "R" emulsion was not quite stable in 0.2 per cent. An experiment made with 0.2 per cent. NaCl gave almost exactly the same titre of agglutination with both sera for both strains, but the control with 0.2 per cent. NaCl of the "R" emulsion was not quite negative. (Table VII.)

TABLE VII.—*Agglutination of the "S" and "R" Forms of B. dysenteriae (Flexner-Y), with Sera made with the same Two Forms, before and after Absorption with Homologous Cultures.*

Serum v. <i>B. dys.</i> 573 "S."	1 80	1 160	1 320	1 640	1 1280	1 2560	1 5120	1 10240	1 20480	NaCl. 0.1%	NaCl. 0.2%	NaCl. 0.4%
Unabsorbed— Emulsion 573 "S"	+++	+++	+++	+++	+++	+++	+++	++	+	-	-	-
„ 573 "R"	+++	++	+	-	-	-	-	-	-	-	-	+
Absorbed 573 "S"— Emulsion 573 "S"	+++	+++	+++	+++	++	-	-	-	-			
„ 573 "R"	++	++	-	-	-	-	-	-	-			
Absorbed 573 "R"— Emulsion 573 "S"	+++	+++	+++	+++	+++	+++	+++	+	-			
„ 573 "R"	++	+										
Serum v. <i>B. dys.</i> 573 "R."												
Unabsorbed— Emulsion 573 "S"	++	+	-	-	-	-						
„ 573 "R"	+++	+++	+++	+++	+++	+						
Absorbed 573 "S"— Emulsion 573 "S"	-	-	-	-	-	-						
„ 573 "R"	+++	+++	+++	+++	+							
Absorbed 573 "R"— Emulsion 573 "S"	-	-	-	-	-	-						
„ 573 "R"	++	+	-	-	-	-						

DISCUSSION.

Variation in the Morphology of Colonies.

I. The method of obtaining variants by selecting colonies of different morphology in plate cultures has been adopted by many writers.

Cobbett and Phillips (1897¹¹) described colonies of two distinct types in gelatine plate cultures of *B. diphtheriae*. They obtained them from six different strains. On gelatine both types remained unchanged through many subcultures, though occasionally some of the larger type appeared on plating out subcultures of the smaller type.

Slawyck and Manacatide (1898³³) described permanent or very persistent variants obtained by the selection of colonies on agar plates of *B. diphtheriae*. The strains obtained differed in the vigour of their growth and in the size and form of their colonies. The present writer has also confirmed these observations. Baerthlein (1912³ and 1918⁴) has published a large amount of work in recent years on the subject of colony variation, and though other workers have not

always been able to confirm his descriptions of colonies, or to find all his variants stable, this may in part be due to the difficulty in satisfactorily describing colonies and to differences in strains, culture-media, etc. Working with *B. typhosus*, *B. paratyphosus*, *B. dysenteriae*, and *V. cholerae* he obtained many variants which were constant in subculture as regards the appearance of the colonies and in some cases as regards the morphology of the bacilli and other characters.

II. It is interesting to try and form an opinion as to the relation of the "S" and "R" forms to the stock cultures which are usually met with in the laboratory. Most strains when examined by plating old cultures can be made to yield both "S" and "R" forms.

If young stock cultures which have been frequently subcultured are plated, the colonies are not of two distinct types but often show intermediate characters, or the colonies may appear to belong for the most part to the "S" form. When inoculated into broth the culture is usually turbid. These stock cultures may agglutinate with both "S" and "R" form sera. It would seem that the colonies from these cultures are made up of bacteria capable of producing both "S" and "R" forms, but that the two forms are not necessarily actually present. In old cultures, however, the "S" and "R" forms appear separately and form separate colonies.

III. No attempt has yet been made to grow the two forms from a single cell. However, since the individuals of the "S" form do not tend to cohere in salt solution emulsions nor in broth cultures, it seems unlikely that isolated colonies of the "S" form are often formed from more than one bacillus; and the "R" form can almost always be derived from old cultures of the "S" form.

If it is assumed that single colonies represent single bacilli, then it seems probable that the two characters are potentially present in all or most of the individual bacilli in young cultures which have been subcultured fairly frequently, and that these characters to a certain extent become segregated in different individuals in old cultures.

This hypothesis of the segregation of the characters in old cultures is also suggested by Ledingham's (1918¹⁶) experience with a reverting strain of *B. dysenteriae* (Flexner-Y). Starting with an isodulcitate fermenting culture he found that a subculture when plated on an isodulcitate neutral-red agar gave (1) red colonies (2) white or pale red colonies which after forty-eight hours became deep red and later produced white papillae. This behaviour suggests that in some of the individual bacilli plated the character of fermentation was feebly represented and did not suffice to make the colony deep red till the second day, by which time more acid had been produced. As the culture got older the character of "non-fermentation" became segregated into individual bacilli which produced white papillae composed of "non-fermenters." Some of the colonies which formed papillae described by Penfold (1910²⁶) suggest the same interpretation.

IV. The third form of Shiga 550 ("RV") met with in the course of these observations presents another problem. It appears to be a further development of the "R" form, since it was obtained in apparently pure culture from a flask inoculated with form "R" of strain 550, and it is normal in its other characters (sugars, indole, motility). Emulsions were agglutinated and precipitated readily in very weak salt solution (0.1 per cent. NaCl). A serum prepared with it agglutinated form "R" of 550 but not form "S."

V. Spontaneous or pseudo-agglutination was noticed and commented on in the years which immediately followed the discovery of specific agglutination by Grubler and Durham, and variant cultures, which showed spontaneous clumping and consequently presented difficulties in the way of the specific agglutination test, have been often described.

Nicolle (1898²⁴) described spontaneous agglutination in broth cultures of *B. typhosus*. Cultures twenty-four hours old were quite clear with a small granular deposit. He found that these cultures had only a slight sensitiveness to serum.

Savage (1901³²) examined the phenomenon of pseudo-clumping in cultures of *B. typhosus*, but found no cause or remedy except that it occurred more frequently in old laboratory cultures, and he did not encounter it in salt solution emulsions from agar nor in peptone-water cultures but only in the presence of broth or meat extract.

Neisser and Friedemann (1904²³) and Bechold (1904⁵) made numerous experiments on the agglutination of sensitised and unsensitised bacteria in different solutions of salts, with a view to studying the relation of specific agglutination to the aggregation and precipitation of particles in inorganic suspensions. However, neither they nor subsequent writers appear to have used a salt solution of diminished concentration to dilute the serum as a means of overcoming the difficulty of testing the serum-agglutinability of spontaneously agglutinating cultures.

Porges and Prantschhoff (1906²⁹) attempted to explain the cause of spontaneous agglutinability, and showed that heating an emulsion of *B. typhosus* to 80° C. (or 65° C. sometimes) removed spontaneous agglutinability, but agglutination by specific serum was also diminished or abolished. In the case of cholera vibrios, heating to 80° in some instances made the emulsion stable in salt solution without preventing agglutination by specific serum.

Paltauf (1912²⁵) in a review of the whole subject of agglutination says that a tendency to spontaneous agglutination by strains of bacteria may be due to the culture medium having been of an unusual or unsuitable reaction, or to the culture being an old one. He also says that if on subculturing on to a suitable medium the emulsion is not stable, and heating the emulsion to 80° C. does not improve it, then the culture must be considered unsuitable for the usual direct agglutination test.

Von Lingelsheim (1913¹⁷) and Sachs-Mücke (1913³¹) described as the "Q" form a variant obtained by plating old cultures of *B. typhosus*. Emulsions in salt solution agglutinated spontaneously. In broth cultures the growth formed a deposit and a surface film leaving the liquid clear. Colonies on plates were large and flat, irregular in outline, dry and coarsely granular on the surface. The emulsions were slimy and the bacilli cohered and showed hardly any motility; otherwise the cultural characters were those of normal *B. typhosus*. Direct agglutination was not tested on account of the spontaneous clumping,

and no attempt at absorption of agglutinins is recorded, but a serum made by immunising with the "Q" form agglutinated other strains of *B. typhosus*. This form persisted in cultures for five years and its peculiar characters survived passage through a mouse. Occasionally some colonies occurred which showed reversion to the normal. Similar forms, but less persistent, were obtained from *B. paratyphosus* B. and *B. enteritidis* cultures. V. Lingelsheim considered that all the characters of the "Q" form might be explained by the presence of a sticky inter-bacillary substance due to a kind of capsule formation.

Baerthlein (1918⁴) states that he obtained inagglutinable variants from all groups of bacteria with which he was working except *V. cholerae*, but he was usually able to obtain sera which agglutinated the original strains by inoculating the inagglutinable variants into rabbits. He does not describe changes of agglutinability such as to suggest a change in antigenic structure of the variant, except in the case of (1) a varying *B. paratyphosus* B. strain and (2) a variant of *B. dysenteriae* "Y."

The "R" form described in this paper corresponds very closely as regards the appearance of the colonies with some of the "inagglutinable" variants of *B. typhosus*, *B. paratyphosus*, and *B. dysenteriae* "Y" described by Baerthlein (1918). One of his *B. paratyphosus* variants was sometimes inagglutinable, and sometimes spontaneously agglutinable and did not absorb agglutinins for the other variants of the same strain. It seems probable that these feebly agglutinating and "inagglutinable" variants described by the above writers were in many cases more or less pure cultures of the "R" form, whose agglutination was not tested in sufficiently weak salt solution. If saline which is too strong is used, the difference between the tubes containing specific serum and the control may be scarcely appreciable. The smallness of the clumps formed by the "R" form has probably confirmed the view taken by these observers that any agglutination which took place was not of a specific nature. This also was the conclusion arrived at by Benians (1920⁷) in the case of an "inagglutinable" strain of Shiga's bacillus, which he described. From his account it appears probable that it was an example of the "R" form.

The "Q" forms described by v. Lingelsheim are undoubtedly variations of the same kind as the "R" forms described in this paper.

Variation in Serological Properties.

VI. In spite of a number of recorded observations on changes in bacteria in respect of the property of agglutination by specific serum, it has usually been held that serological properties are constant and never vary, although variability has been accepted as regards other properties. The antigen peculiar to one kind of bacterium has come to be regarded as an almost unchangeable constituent of the bacterium which always produces the same antibody response, as in the case of a pure protein, such as a particular kind of egg albumen.

Although some bacteria are remarkably constant as regards their antigenic constituents and the corresponding antibodies, e.g., *B. typhosus*,

the generalisation is perhaps hardly justified in so complete a form on experimental grounds. Loss of agglutinability by a culture has perhaps too exclusively been ascribed to a failure in the second non-specific part of the reaction.

The changes described in respect of serum properties have usually been in the direction of loss of agglutinability, but some instances of the additional loss of antigenic power and of ability to fix complement have been recorded. Records of changes such that a variant produces an antibody which does not react with the parent culture or with other variants from the same stock are rare.

Bordet and Sleswyk (1910³⁴) described the manifestation of entirely different serological properties by the whooping-cough bacillus according to whether it was grown on blood-agar or on ordinary agar. Although the difference which they record is simply ascribed to the change in culture medium, the need for gradually accustoming the cultures to the new medium suggests the selection out of a variant.

Sobernheim and Seligmann (1910^{34,35}, 1911³⁶) gave evidence of interchange of characters between members of the *B. paratyphosus* and *B. enteritidis* groups of bacteria and of the occurrence of intermediate forms. These changes concerned agglutination, absorption of agglutinins, and antigenic properties.

Baerthlein (1918⁴) described (A) a mutant of *B. dysenteriae* Y, which was "inagglutinable" and did not absorb specific agglutinins nor fix complement with a serum (titre 1/10,000) derived from a normal strain; (B) a strain of *B. paratyphosus* B. which underwent still more extraordinary changes, since it was altered eventually in almost all its characters. The changes are stated to have taken place by intermediate steps. This strain first produced six varieties of colony. Two of these (1) and (4) varied as regards agglutinability and they did not absorb from a specific serum the agglutinins for the other variants. Variant (4) formed very large colonies with jagged outline and was inagglutinable; a serum made from it agglutinated the homologous and all the other variants. Variant (1) also formed large, jagged colonies; it was non-motile and sometimes inagglutinable, and at other times agglutinated spontaneously. When inoculated into a rabbit it produced a serum which did not agglutinate itself nor the other variants. In respect of sugar reactions it agreed with the parent strain. Variant (1) was very constant and irreversible for three and a half months, but after five and a half months a further variation made its appearance. This sub-variant fermented glucose and mannite without gas, but it still turned litmus milk blue in forty-eight hours after a preliminary acid stage. With paratyphoid B. serum (1/3000 titre) it agglutinated to 1/500 or slightly higher, and with *B. typhosus* serum (titre 1/20,000) to 1/10,000. Baerthlein appears to consider these changes as almost equivalent to a transmutation from *B. paratyphosus* to *B. typhosus*.

Van Loghem (1919¹⁸) describes variations which occurred in a culture of *B. paratyphosus* B. after it had been used as a type culture for nine years. The first changes noticed were that it formed indole and agglutinated irregularly. After three more years of subculture it was found that a large proportion of the colonies formed indole and that gas production was diminished. Further variants from the same strain agglutinated feebly with paratyphoid serum and up to half titre with typhoid serum. V. Loghem looks on variability as a characteristic of *B. paratyphosus* B., and does not consider his observations as evidence that one species is being transformed into another.

Mellon and Anderson (1919²²) claim to have made separate sera from the bacillary and spore forms respectively of *B. subtilis* which have quite distinct

serological properties, and state that cross agglutination does not occur. If this is confirmed, the results may have some analogy to the different properties of the "S" and "R" forms described in this paper.

Hort (1920¹⁵) describes remarkable forms of *B. typhosus* which he believes to have a relation to a special method of reproduction. He finds these forms sometimes associated with a different behaviour towards agglutinating sera.

VII. The correlation of the variations in agglutination by salt and by serum is of great assistance in enabling one to select colonies of serological variants. The coincident change in both characters (agglutination by salt and by serum) is perhaps not fortuitous.

It has been shown by Beintker (1912⁶), Beniasch (1912⁸) (and others) that these two properties (agglutinability by electrolytes and by specific serum) are to some extent related. Moreover, they considered that the substance in the bacteria which was concerned with agglutination by electrolytes and by specific serum was the same in both cases since, they found, that emulsions which are inagglutinable by acid are also inagglutinable by serum.

Arkwright (1914²) confirmed the close association of the properties of agglutinability by serum and by acid but considered the identity of the agglutinable substances unproven.

The two phenomena do not run exactly parallel, but there is a large amount of evidence tending to support this view.

Eisenberg (1919¹³) examined a long series of cultures of different kinds of bacteria, and again called attention to the close connection between agglutinability by specific serum and by electrolytes.

Other Variations.

VIII. Other variations as regards the fermentation of sugars, etc., pigment formation, hæmolytic action, toxin-production, virulence, etc., have been frequently recorded and are of importance in connection with the subject of this paper, in so far as they have been carefully observed, because they indicate the wide range of the tendency to variation.

Variations in Fermentation.—The occurrence of "mutation" in respect of the production of acid and gas from carbohydrates and alcohols has been worked at very carefully and the processes much elucidated by Massini, Twort, Müller, Penfold, Revis, Ledingham, and other writers who have observed and discussed the degree of permanence of the mutations and the occurrence of reversion. For reference to papers on this subject, see Penfold (1910²⁶ and 1912²⁷).

IX. *Relation of Sliminess of Cultures to Morphology.*—The swollen-looking, large, broad, irregularly shaped bacteria sometimes showing bud-like and branch-like processes which I have met with in films from some 24-hour agar cultures of the "R" form of *B. dysenteriae* (Shiga) are very like the forms of *B. typhosus* figured by Hort (1920¹⁵), who considers them to be of special reproductive importance. In my cultures these peculiar forms have always been associated with sliminess of the emulsions. The latter property appears to be due to a change in the internal composition of the bacilli and not to a capsule formation as was the case in the variant of *B. coli*, which formed jelly-like colonies

described by Revis (1910³⁰), and the "mucoid" forms of *B. paratyphosus* B. and *B. dysenteriae* described by Fletcher (1918¹⁴).

The special agglutination properties of the "R" form may have some relationship to the swollen forms and the presence of slimy material.

Ledingham has suggested that the mucus-like substance in the cultures may be the explanation of the peculiar type of agglutination exhibited by the "R" form both when under the influence of specific serum and of salt alone. V. Lingelsheim (1913¹⁷) considered the peculiarities of his "Q" form to be entirely due to the sliminess of the cultures. In my cultures, however, the special kind of agglutination of the "R" form by serum appears to be more nearly associated with agglutinability by salt than with the presence of slime which seems to be an independently variable character.

X. Variations of bacteria which have arisen in the animal body have also been recorded. Sørensen (1912³⁷) and Arkwright (1913¹) both relate instances in which organisms of the *B. coli* group lost the power of forming gas in the human bladder. The inagglutinable variant of *B. dysenteriae* (Shiga) described by Benians (1920⁷), was obtained from an inoculation abscess in a guinea-pig.

Nature of the Variation into "S" and "R" Forms.

XI. The views of biologists as to the nature of variation are very unsettled at the present time, but since it seems quite justifiable to consider bacteria as asexual throughout their life history, the explanation of variation as due to cross fertilisation need not be considered. The accounts of conjugation and of the union of several individuals by Löhnis and Smith (1916²¹) at present need confirmation.

There are several possible explanations of the origin of the "S" and "R" forms which may eventually be partially settled by single cell culture.

(1) The "R" form might be due to a contamination with an entirely different micro-organism. This seems unlikely, since it would involve the hypothesis that two entirely distinct bacteria were constantly present in stock cultures of different strains and were practically inseparable.

(2) It may be held that the "S" and "R" forms pre-exist in all cultures as different strains or elementary forms of the same "species," and that many such strains habitually live side by side. This hypothesis would be available to account for "variations" of the same organism in other directions, *e.g.*, pigment production, fermentations, sliminess, agglutination by salts and by specific serum, etc. Such an interpretation would be consistent with the hypothesis of multiple antigens put forward by Durham (1901¹²).

A somewhat analogous explanation of polymorphism in species of higher plants, *e.g.*, cereals, is stated by de Vries (1909³⁹). According to

this view certain apparently new mutants or variants, which when selected by the plant-breeder prove constant and breed true, have in reality been for long present amongst the many elementary forms which together make up the Linnæan species.

This explanation has been advocated by Brierley (1919¹⁰) when dealing with the meaning and limits to be attached to the idea of species in fungi and bacteria. He considers that the claim that species are constantly shifting and changing whilst under observation is *a priori* highly improbable. This line of reasoning appears to prejudge the question of the origin of new heritable forms or species in unicellular non-sexual organisms, like bacteria, whilst under observation.

It appears to have been settled as far as is possible at present by methods of single cell culture that some forms of variants can arise from single bacteria and are not explicable on the theory of multiple strains. Experiments with *B. coli mutabilis* by Müller (1909), Penfold (1912²⁷), and others, by Burri's method are among the most important evidence on this point; and other forms of variation may be tested in this way.

(3) The two forms "S" and "R" might be merely "modifications" due to the environment, but in this case a change of environment, *e.g.*, a different culture medium, should soon re-establish the normal; and the two forms when grown on identical medium should at once approximate. This, however, does not occur.

(4) The different characters of the "S" and "R" forms might be due to "fluctuation" or "variation within the limits of the species." By this is meant such changes as are limited in kind and degree, are obtainable from all strains of the organism, and have a very strong tendency to reversion.

This notion of variation is of the same kind as that put forward by R. Müller (1909) and Penfold (1912²⁷), as applicable to certain "mutants" observed by them. They held that these variations did not afford evidence of a tendency to transmutation from one species to another, but were really special changes which were characteristic of the species and might aid in its identification.

It is difficult to decide whether this is a suitable category in which to place the "S" and "R" forms, since the dividing line between "fluctuations" and "mutations" (the two chief kinds of variations described) appears to be a very narrow and uncertain one. The permanence of the "S" and "R" forms and their definite characters are opposed to the view that they are fluctuating variants, but on the other hand the constancy with which they can be obtained from different strains shows a strong inherent tendency of the "species" to vary in this way. Van Loghem (1919¹⁸) appears to attach a very wide significance to the term "variation within the species." When discussing his *B. typhosus*-like variant of *B. paratyphosus* B. he seems to imply that all the characters of a member of the *B. paratyphosus* must

change, till it is absolutely indistinguishable from the members of another species and has become fixed, before he would admit it as a case of mutation. Such a change would more aptly be called a transmutation.

In the category of "fluctuations" should probably be included the kind of change described by Löhnis and Hanzawa (1914²⁰), Löhnis and Smith (1916²¹), and Hort (1920¹⁵), if the hypothesis of these writers were to be accepted that such changes were of a cyclical nature. The further question as to whether the processes concerned include sexual phenomena as they suggest, may be left for the present till further confirmation is forthcoming.

Löhnis and Smith's paper suggests, however, that the changes with which they are dealing are in part persistent with no very strong tendency to revert, and in this they agree with the "S" and "R" forms.

(5) The term "mutant" may perhaps be used appropriately for the "S" and "R" forms. By a "mutant" is meant a decided and persistent variation which may be progressive, whether by loss or acquirement, *i.e.*, may lead to further changes such as are held on the Darwinian hypothesis to lead to new species by selection. There does not seem to be anything definitely against these forms being classed as "mutants." Still the fact that they arise from so many strains must make one hesitate unless further evidence of progressive change is forthcoming, than is afforded by the "RV" form referred to above.

(6) The manner of origin of the "S" and "R" forms does not affect the fact that they are both present in many cultures which are believed to be pure, and that their special characters concern the agglutinating, absorbing, and serum-producing properties of these strains.

XII. Whatever view is taken regarding the origin of the "S" and "R" forms they undoubtedly readily arise under artificial surroundings. It is clear, therefore, that selection must play a very important part in explaining the normally uniform characters of such well-known bacteria as *B. typhosus* and *B. dysenteriae* (Shiga). The human body infected with dysentery may be considered a selective environment which keeps such pathogenic bacteria to the forms in which they are usually encountered.

Occasionally, however, colonies suggesting the "R" form occur on the first plates used for isolating from the faeces, and Benians (1920⁷) has recently described a spontaneously agglutinating strain of *B. dysenteriae* (Shiga) which was isolated from an abscess in a guinea-pig produced by inoculating a normal strain.

It must, moreover, be remembered that strains which do not agglutinate spontaneously, and which consequently form smooth colonies and are agglutinated in easily visible clumps by serum, are sought and

selected by bacteriologists, and that colonies of unusual appearance and cultures which make unstable emulsions are as a rule discarded and neglected. In this way the strains most commonly studied and held to be normal and typical have been artificially selected by man.

That variants and mutants do not become fixed more frequently is perhaps due to the environment (whether in the body in the case of pathogenic bacteria, or in the test-tube in the case of artificial cultures) being rather rigid and stereotyped, so that "natural" selection tends to keep the strain to the form in which we know it. In the case of the "S" and "R" forms (1) the shape and appearance of the colonies on agar and (2) the manner of growth in broth are prominent features acting as indicators of the changes in agglutinability by salt which enable artificial intentional selection to act, and to obtain apparently pure cultures of the two forms.

Whether varieties of bacteria in any direction can be detected, obtained pure and fixed in the laboratory, probably depends largely on whether the variation is such that it is accompanied and revealed by some readily noticed change, which acts as an indicator of the variation and assists intentional selection. Such an indicator is most useful for the purpose if it can be seen in colonies on a plate culture. For instance, the pigment variations of cocci, of *B. prodigiosus* or *B. pyocyaneus*, the red and white colonies on MacConkey's medium indicating fermentation properties, or the smooth and rough colonies associated with variations in agglutination.

XIII. Variation of the kind under discussion appears to be liable to occur in many directions, but there seems to be no reason why it should occur in more than one direction at once, and transmutation in artificial culture of one pathogenic organism into another as regards all its characters seems therefore to be highly improbable. It seems likely that such a change if it were possible would require the complicated environment of the animal body reacting to a more or less pathogenic organism, in fact the conditions under which all well-defined strains of pathogenic organisms may be supposed to have risen.

XIV. It seems probable that even considerable changes may be liable to occur in cultures without disturbing the ordinary conclusions arrived at in the routine practice of bacteriology, so long as well-known methods are used and new media and technique are not introduced without due caution and careful study of their effects. The characters of fairly frequently subcultured cultures tend to be ill-marked and indefinite as regards the commoner variations, at least so far as the "S" and "R" characters are concerned. That is, the stock cultures usually exhibit mixed characters, and a mixture of forms may actually be present.

XV. It is obvious that before accepting large changes which affect simultaneously important properties such as agglutination by specific

serum, absorption of agglutinins and complement fixation, such as Baerthlein (1918⁴) claims for one of his variants of *B. dysenteriae*, great care in using the technique of isolation and subculture is required of the observer and in applying tests with regard to the other properties of the variants. Indeed, if many characters are said to have varied at the same time such as gas-production, indole-formation and relative agglutinability with typhoid and paratyphoid sera, as in the case of the "mutant" of *B. paratyphosus* B. described by van Loghem (1918¹⁵), the conclusion must remain in doubt unless the variation can be obtained repeatedly.

CONCLUSIONS.

(1) Eight out of the nine strains of *B. dysenteriae* (Shiga) examined have been made to yield two forms ("S" and "R"), which remain constant in broth when subcultured weekly; they have different cultural characters in broth and on agar, but are identical and resemble the stock culture as regards sugar reactions and the absence of indole production, and of motility. The remaining strain only grew in the "R" form.

(2) The "S" form makes a good stable emulsion in salt solution, and in broth cultures causes uniform turbidity. The "R" form agglutinates in 0.85 per cent. solution of sodium chloride, and in broth cultures it forms a deposit leaving the liquid clear. Weaker salt solution must therefore be used for agglutination experiments with specific sera.

(3) Many cultures of bacteria which agglutinate spontaneously when emulsified in 0.85 per cent. solution of sodium chloride form good stable emulsions in weaker salt solution, *e.g.*, 0.42 per cent., 0.21 per cent., or 0.1 per cent.

(4) Agglutination tests with specific sera and "spontaneously agglutinating" strains can be carried out perfectly satisfactorily in salt solution of $1/2$ to $1/8$ the usual concentration (*i.e.*, 0.42 per cent. to 0.1 per. cent. sodium chloride). A normal (not spontaneously agglutinating) culture should, if possible, be also tested in the same strength of salt solution, so as to ascertain the titre of the serum in this concentration of salt.

(5) The "S" form, when agglutinated by specific serum, forms large clumps. The "R" form makes small clumps and the deposit is readily shaken up into a turbid suspension.

(6) The two forms differ very decidedly in their agglutinating, antigenic, and absorbing properties with specific sera.

(7) The "S" forms obtained from different strains of *B. dysenteriae* (Shiga) resemble each other serologically, but are distinct from all the "R" forms, and the "R" forms of different strains appear to have little relation to any "S" forms, but to be closely related to each other.

(8) "S" and "R" forms showing similar properties have been obtained from one strain of *B. dysenteriae* (Flexner-Y).

(9) Similar "S" and "R" forms have been obtained from several strains of *B. typhosus*, but the serological differences between the two forms were less distinct than in the case of *B. dysenteriae*, in the case of the one strain of *B. typhosus*, in which specific serum reactions were tested.

(10) Forms resembling the "S" and "R" forms as regards cultural characters and "spontaneous" agglutination have also been obtained from cultures of *B. paratyphosus* B., *B. enteritidis* (Gaertner), and other members of the *B. coli-typhosus* group, but the serological characters have not been examined by means of specially made sera.

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DESCRIPTION OF PLATE II.

FIG. 1.—Agarplate showing colonies of *B. dysenteriae* (Shiga) of two kinds: (1) smooth round domed ("S" form) and (2) flattened with irregular surface and margins ("R" form). ($\times 6$ diams.)

FIG. 2.—Agar plate showing colonies of the "S" form of *B. dysenteriae* (Shiga) No. 550 I. 24-hours old culture. Typical smooth colonies. ($\times 6$ diams.)

FIG. 3.—Agar plate showing the commonest kind of colonies of the "R" form of *B. dysenteriae* (Shiga) No. 550 II. which gives a slimy emulsion. 24-hours old culture. Typical flattened semi-opaque colonies with irregular margins and slightly dull surface. ($\times 6$ diams.)

FIG. 4.—Culture on agar plate of *B. dysenteriae* (Y) No. 573 III. 24 hours old, showing two kinds of colonies of the "R" form: (1) flattened with irregular margins and slightly irregular surface; semi-opaque. (2) Larger colonies, flat, very thin, with very uneven coarsely granular surface, and thin edges.

FIG. 5.—Film from 24-hours culture on agar of the "S" form of *B. dysenteriae* (Shiga) showing uniform short bacteria. ($\times 2000$.)

FIG. 6.—Film from 24-hours culture on agar of the "R" form of *B. dysenteriae* (Shiga) from colonies like those in Fig. 3, which give a slimy emulsion. Very irregularly shaped, wide bacteria sometimes with lateral bud-like knobs; mostly deeply stained, but some ill-stained and shadowy. ($\times 2000$.)

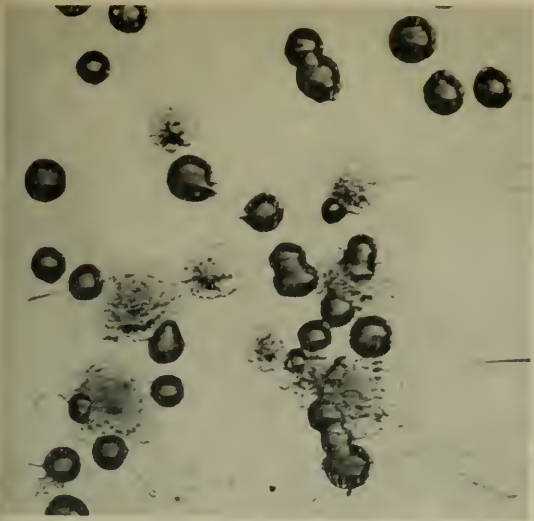


FIG. 1.

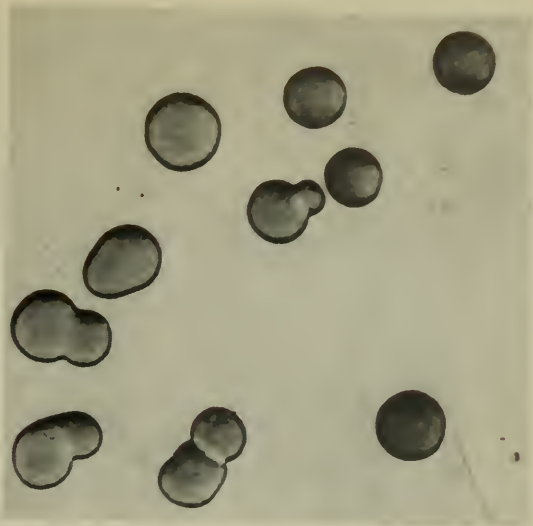


FIG. 2.

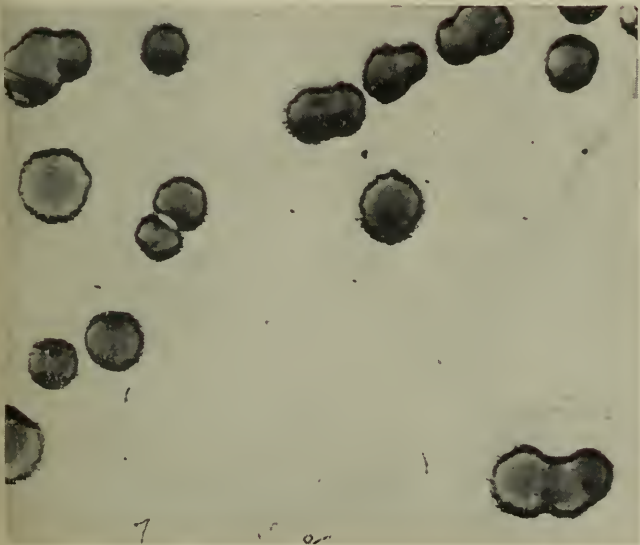


FIG. 3.

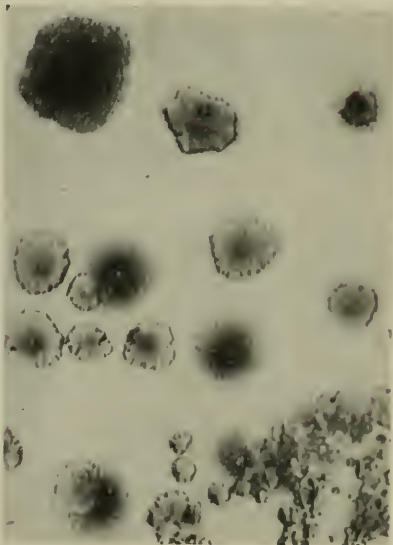


FIG. 4.

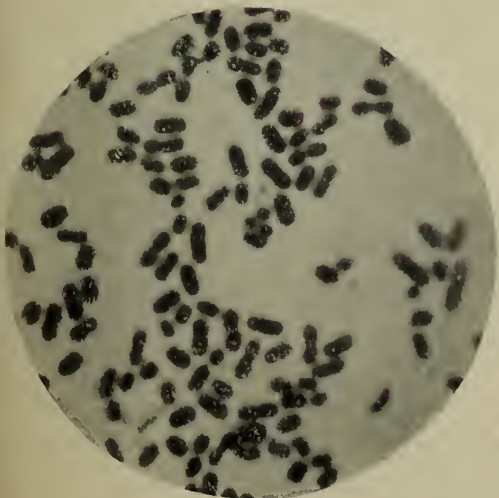


FIG. 5.

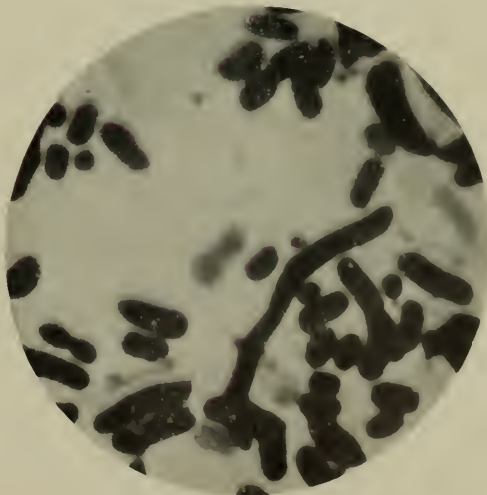


FIG. 6.

A BACILLARY INFECTION OF THE COPULATORY
APPARATUS OF *PEDICULUS HUMANUS*.

BY J. A. ARKWRIGHT AND A. BACOT.

(From the Lister Institute of Preventive Medicine.)

IN the paper on the association of *Rickettsia* with Trench Fever, by Arkwright, Bacot and Duncan (1919) mention is made of a bacillary infection of the excreta and guts of lice (*Pediculus humanus*). A Gram-negative cocco-bacillus was isolated in pure culture. It was non-motile, fermented glucose, mannite and lactose very slowly, and formed acid and clot in milk in about 14 days.

It was not pathogenic for guinea-pigs. When plated from pure culture, rough and smooth colonies were formed similar to those which are known to occur in bacilli of the *B. coli* group.

Continuation of our work with *Pediculus humanus* has placed us in possession of further information concerning the relation of this bacillus to its host.

Sections of lice from a stock which has been inbred for nearly five years show a high percentage infested with this parasite. It is probable that crowding together in boxes for so long a period has caused these lice to be more uniformly infected than wild ones. In male lice the cocco-bacilli are seen in the folds of the *vesica penis*, whereas in females they occur in the vaginal orifice and passage leading to the ovaries¹. No evidence that the gut is involved has been obtained from the very numerous sections examined.

Preliminary culture experiments with the guts of 24 lice taken at random from the stock were carried out. The insects were washed in 2 per cent. lysol followed by rinsing in sterile salt solution and the alimentary tract was dissected out on flamed slides with sterilised needles. Each specimen was transferred by means of a platinum loop to a separate nutrient agar tube and placed at the lower part of the slope.

Half the tubes were placed under anaerobic conditions, six at 35° C., and six at 26.5° C.; the remaining twelve were incubated aerobically at similar temperatures. Growth took place in seven of the twelve kept at the lower temperature, including both aerobic and anaerobic tubes. Subsequently five of the remaining tubes, which had been transferred to the cooler incubator, also produced growths. All the cultures appeared similar both to the naked eye and on microscopic examination. Subcultures were plated from the tubes and found to be pure. Both subcultures and plates were made with ordinary

¹ See Nuttall (1917), *Parasitology*, ix. 293 for description of copulatory apparatus of ♂ and ♀.

nutrient agar and were kept aerobically at 35° C. Later attempts to cultivate direct from the dissected guts at 35° C. have proved successful, so that the failures at this temperature in the first trial must be attributed to some chance circumstance. It may be noted here that to dissect out the alimentary system of the louse without contaminating it with organisms infesting the copulatory apparatus would be very difficult if not impossible, as the pressure of the dissecting needles would tend to release the valves which close the openings to both the male and female organs. The later series of trials was therefore planned in order to decide whether the gut was infected or not.

Six males, six females, and a like number of nymphs (in which the copulatory apparatus is not yet developed), were taken from the stock and treated in the same manner as in the previous experiment except that the two terminal segments were left adhering to the gut.

This experiment resulted as follows: six of the tubes containing the female and five of those containing the male guts produced growths of similar appearance to those in the previous experiment. Microscopically, when stained by Gram's method, they all showed similar cocco-bacilli, apparently in pure culture. Subcultures on plates from two tubes selected at random from among the eleven infected supported this assumption. As regards the tubes containing the dissections of the guts of nymphs only one showed a growth, and this was obviously contaminated as the growth started apart from the introduced gut, was of an entirely different appearance and was composed of Gram-positive bacteria.

Cultivation therefore strongly supports the microscopic evidence obtained from sections. We conclude that this bacterium may be correctly termed a parasite of the copulatory apparatus of *Pediculus humanus* and we propose for it the name of *Bacillus pediculi*.

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AN HEREDITARY *RICKETTSIA*-LIKE PARASITE OF THE BED BUG (*CIMEX LECTULARIUS*).

BY J. A. ARKWRIGHT, E. E. ATKIN AND A. BACOT.

(From the Lister Institute of Preventive Medicine.)

(With Plate II and 1 Text-figure.)

THE KNOWN SPECIES OF *RICKETTSIA*.

THE forms under consideration resemble *Rickettsia prowazeki*—the supposed cause of typhus fever—which occurs in lice that have fed on typhus fever patients.

It may therefore not be amiss to describe briefly the organisms which have been grouped by various workers in the same category as *Rickettsia*.

The general characters of “*Rickettsia*” may be summarised as follows:

(a) Bodies of minute size, usually 0.5μ in diameter or less, of round or diplococcal shape, though very minute bacillary and even thread-like forms occur.

(b) Though resembling very small bacteria in general appearance, they stain much less readily than ordinary bacteria but can be coloured with Giemsa’s stain; they are readily decolourised by Gram’s method.

(c) Absence of motility.

(d) Resistance so far, with one exception, to attempts made to cultivate them on artificial media *in vitro*.

(e) Their occurrence in very large numbers in the gut and in some cases in other organs of blood-sucking insects.

The known organisms apparently belonging to this group are:

(1) The organism found in the tick—*Dermacentor venustus*—the invertebrate host of the parasite causing Rocky Mountain spotted fever.

This was first described by Ricketts and has since been very thoroughly studied by Wolbach (1919) and called by him *Dermacentroxenus rickettsi*.

It is found in very large numbers in the alimentary canal, salivary glands, muscles and other organs of the tick and is passed on to the next generation of ticks. Similar forms have been described in the blood of men and other animals infected with this disease; Wolbach records its regular occurrence in the tissues of the mammalian host. This organism varies considerably and according to Wolbach, has three distinct morphological forms.

(2) *R. prowazeki* described in detail by da Rocha-Lima (1916) and previously by Ricketts (1909) and Sargent, Foley and Vialatte (1914). This is found in

masses inside the cells of the mid-gut of *Pediculus humanus* after the latter has fed on typhus patients. Although often present in large numbers free in the gut, it appears to multiply inside the epithelial cells. Wolbach and Todd (1920) have described similar forms in the tissues of human sufferers from typhus. The organism varies considerably in shape and size; usually it is round, diplococcal or oval, but often resembles short bacilli; thread forms also occur.

(3) *R. quintana* or *wolhynica*, first described by Toepfer (1916) as the cause of trench fever, occurs in enormous numbers in the lumen of the gut of lice (*P. humanus*) which have fed on trench fever patients. It is more constantly rounded, oval or diplococcal than *R. prowazeki* and is not known to have occurred in thread forms. This species also stains a deeper purple by Giemsa's method. Most writers agree that the organism does not occur inside cells. It may be identical with *R. pediculi*.

(4) *R. pediculi*, first described by Munk and da Rocha-Lima (1917) as an occasional inhabitant of the gut of normal (uninfected) lice and subsequently said by them to be present in trench fever lice. They say that it is indistinguishable from *R. quintana*. It seems probable that the supposedly normal lice in which the parasite has been found had fed upon convalescents from trench fever whose disease had not been diagnosed.

(5) A species of *Rickettsia* found in lice which had fed on persons suffering from "war nephritis" was described by Toepfer (1917), who believed that he could distinguish it from *R. prowazeki* and *R. quintana* by its morphology. This has not been confirmed.

(6) A form of *Rickettsia* which Munk and da Rocha-Lima (*loc. cit.*) found occasionally in a few batches of lice which had either fed on normal persons or on trench fever patients. Munk and da Rocha-Lima stated that the organism was larger and stained more deeply than *R. prowazeki*, that it occurred not only in the lumen of the gut but also inside the cells lining the alimentary canal, in contrast to *R. pediculi* and *R. quintana*. These writers also stated that the organism damaged the cells of the gut and interfered with the powers of the insect to digest blood; they believe it to be a special parasite of the insect and not to be associated with human disease. If Munk and da Rocha-Lima are correct in this opinion their organism resembles the next species and that found in *A. lectularius*, which is described in this paper, so far as restriction to the invertebrate host is concerned.

(7) *R. melophagi*. This organism has been described by Noeller (1917), Sikora (1918), Jungmann (1918), and others, who say it is constantly present in the middle part of the stomach of *Melophagus ovinus* (the sheep "ked"), in large numbers in the older adults, in smaller numbers in young adults, and even occurs in pupae or in very young adults which have not yet sucked blood. The parasite is, they believe, hereditary in the "ked" and not derived from the sheep. The forms described are slightly larger than those of *R. pediculi* or *R. prowazeki*, round, oval or diplococcal in shape. Threads have not been

observed and this organism lies on the surface of the epithelial cells. It has been cultivated on blood agar by Noeller and Jungmann.

(8) *R. ctenocephali* was found by Sikora (*loc. cit.*) in 20 out of 100 cat fleas examined. It is said to be very like *R. quintana*.

(9) The last-named writer found similar forms in smears from the Malpighian tubes of a mouse flea.

RICKETTSIA IN *C. LECTULARIUS*.

Our knowledge of the form we are about to describe, which has hitherto apparently escaped attention, resulted from an attempt to infect bugs with the virus of trench fever by feeding them on patients suffering from this disease. Examinations of smears made from the guts of these insects showed in nearly every case thread-like "bacteria" with some admixture of shorter, rod-like forms (Plate II, fig. 2).

Stained with Giemsa's stain there appeared to be an outer sheath which took the eosin rather lightly while in the interior were granules or groups of granules which stained more deeply and of a purplish hue.

In some cases these smears also showed numbers of small deeply staining coccal or diplococcal bodies which, although slightly larger than *Rickettsia* found in gut and excreta smears of lice that had been fed on trench fever patients, were still sufficiently like them in size and general appearance to suggest that they might be the same species modified by development in the body of an unusual host. The fact that these bodies were not detected in any of the earlier smears from control bugs, although the thread-like forms above described were present, led to unsuccessful attempts to infect two volunteers with the emulsified guts of infected bugs in which the *Rickettsia*-like bodies were present. The examination of further control smears showed, however, that these minute bodies were also present in bugs that had fed only on normal men.

Suspicion of a relation between the rod and thread forms and these minute bodies arose owing to the occurrence of darkly stained granules in the rod and thread forms.

That these minute bodies frequently escaped our notice in smears which showed the long bacterial forms, is due partly to their small size, which makes it difficult to recognise them unless a considerable number are present in a single field; the same difficulty has been found in the case of the *Rickettsia* of trench fever. The fact that their distribution is often very localised is, however, the chief cause of difficulty in their detection. Whereas the bacillary forms are nearly always generally distributed in smaller or larger numbers in smears of well teased guts or Malpighian tubules, the minute forms, even when present in large numbers, are frequently only to be found in proximity to fragments of the gut or tubules. In some smears they were only seen where they chanced to be escaping from the ruptured end of the gut or a

tubule and no doubt the stage of the parasite in the infected cells affects the readiness with which it is found.

MOTILITY.

A study of fresh wet preparations by either transmitted light or dark ground illumination failed to reveal any signs of motility.

STAINING.

The most effective staining process tried is the slow method with weak Giemsa stain as generally employed for Rickettsia work, viz. 1 drop of stain to 1 c.c. of distilled water applied for 10–24 hours. All the forms are decolourised by Gram's method; the rod and thread forms are only faintly stained by the fuchsin counter stain. Strong fuchsin produces only a slight effect on the long forms and does not satisfactorily stain the minute coccal or diplococcal bodies. With Giemsa's stain the parasite does not react uniformly, the character of the tissues in its immediate vicinity apparently exercises an influence on the staining process. Where infected cells have been ruptured immediately prior to fixation or if the parasite is included in unbroken cells the long forms stain more intensely than when they are free. In the former situation the stained organism is purple, or if red, shows a gradation to the purple of the internal granules instead of an abrupt transition. It would appear that once the bacillary forms are removed from their natural habitat they rapidly undergo some change which causes them to stain badly.

CULTIVATION.

Attempts were made to cultivate the organism on artificial media. All the ordinary media were tried, aerobically and anaerobically, and in addition Dorset's egg medium, Noguchi's medium for spirochaetes, Krumwiede and Pratt's (1913) semi-solid medium which is successful in the cultivation of *B. fusiformis*, and the body juices of a lepidopterous pupa (*Hadena oleracea*), but in each case without a positive result.

In view of the remote possibility of the organism being a stage in the developmental cycle of a spirochaete, some mice were inoculated by scarification and subcutaneous injection with infected Malpighian tubes of the bug but no spirochaetes appeared in the blood.

EVIDENCE OF THE HEREDITARY CHARACTER OF THE PARASITE.

In the absence of evidence that the parasite had any second host, theoretical considerations based on the feeding habits of *Cimex lectularius* suggested that the organism must be passed on through the egg. An examination of smears made from the alimentary system of newly hatched unfed bugs showed that the latter were infected. Eggs washed in 2 per cent. lysol for five minutes and then in sterile salt solution contained both rod and thread forms, the

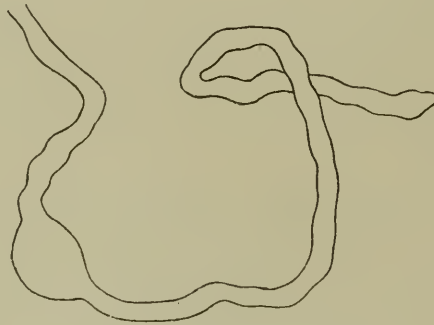
former being more common. This was also the case in smears of unhatched bugs extracted from the egg. Smears of eggs showing the early developmental stage of the bug (as a rule these eggs have to be dissected out of the ovary or made into smears soon after laying), contain immense numbers of more or less well-stained granules, usually poorly defined, but in some cases of distinct *Rickettsia* form and sharp in outline (Plate II, fig. 7); this feature may characterise the greater part of the smear. More frequently, however, it is confined to small patches in what are otherwise areas of ill-defined granules. Since we failed to cultivate the organism we cannot tell whether the ill-defined granules are related to the parasite or whether they are merely particles of protein. The sharply outlined forms are, we believe, undoubtedly a stage in the life-cycle of the organism. The minute bacillary forms that are present among the coccal and diplococcal forms are much more numerous in some smears than in others. Smears of the guts of embryo bugs at a late stage of development extracted from the egg, in some cases show masses of the minute forms deeply stained and clearly defined, lying within the lumen of the gut, where they have presumably been isolated as a part of the detritus of the embryonic process.

In order to obtain additional proof of hereditary infection, ovaries were dissected out of females that had been washed in lysol and then in sterile salt solution. Smears made from eggs extracted from these ovaries usually showed a few threads with some admixture of the shorter bacillary forms in addition to the granular infection alluded to above.

INTRACELLULAR MULTIPLICATION AND DEVELOPMENT.

Apart from its presence in the undifferentiated egg mass, the development of the parasite seems to be entirely intracellular. No evidence has been obtained of its multiplication in the lumen of the gut or in the body cavity where, however, thread forms have been found. Smears of teased embryos taken from developing eggs and of various organs from older bugs including the ovaries, testes, organ of Berlese and Malpighian tubes show minute coccal, diplococcal and lanceolate forms. These latter are slightly larger than the coccal and diplococcal (*Rickettsia*) forms and stain red instead of purple. Clusters of these red staining forms (Plate II, fig. 1), sometimes accompanied by a few of the *Rickettsia* forms, occur in the cytoplasm. They are best observed, individually, when the infected cell has been ruptured in such a manner as to spread its contents without breaking its nucleus. Very many examples of intermediate forms between these bodies and the longer bacterial forms have been observed; some clusters showed the lanceolate forms together with their various stages of growth into rods (Plate II, fig. 3). The intermediate forms were very often curved. Growth of the rods into threads is shown in almost every smear but apparently only in the enlarged cells of the Malpighian tubes do the threads attain their full length (Plate II, fig. 8).

The minute forms are also to be seen in some of the cells of teased guts in small numbers. In the cells of the seminal pocket on the ventral side of the fourth abdominal segment of adult females, known as the organ of Berlese¹, and in the cells of the Malpighian tubes clusters of the lanceolate forms are of frequent occurrence, sometimes very large ones (Plate II, fig. 4), while the *Rickettsia* forms are frequently present in immense numbers in the enlarged cells of the tubes in company with the thread forms. There would seem, however, to be a distinction between the course of intracellular development in the two organs in that, so far as present observation goes, threads and rods are comparatively scarce in the organ of Berlese, whereas they are exceedingly plentiful in the infected Malpighian tubes, generally in excess of the *Rickettsia* bodies. The longer bacterial forms are often present in the cells of certain portions of the gut wall, but they are never very numerous in any one cell.



Text-figure. Swelling in Malpighian tube of *Cimex lectularius* due to infection by the parasite.
Camera lucida drawing from unmounted dissection of adult female.

Within the cells of the Malpighian tubes the multiplication of the parasites becomes so great that individual cells are swollen to more than twice their normal diameter. This has only been observed in the larger nymphs or adult bugs. It is not uncommon to see two or more tubes each with one or more swellings in the same insect and as the tubes at these points may be two or even three times their normal diameter (Text-figure) the infection is quite apparent under the dissecting microscope.

The enlarged cells in fresh preparations are more transparent than normal ones, sometimes showing a faint greenish hue in the centre of the swelling, and when opened they are found to be packed with the thread forms and granules in varying proportions (Plate II, fig. 8). In sections such cells have a bird's nest appearance owing to the intertwining of the long bacterial

¹ The process of fertilization in the bed bug differs very remarkably from that usual among insects. The penis is inserted into an opening in the right side of the ventral plate of the fourth abdominal segment of the female. Immediately above this opening lies a spherical organ consisting of a mass of cells known as the organ of Berlese. This organ has no duct or outlet and the spermatozoa penetrate through the mass of cells composing the organ into the body cavity, finding their way to the oviducts and fertilizing the eggs *in situ*. The development of the embryo is frequently well advanced before the egg is laid. (For further details see Craig (1915), *Indian Journal of Medical Research*, 11, No. 3, pp. 698-705.)

forms (Plate II, fig. 6). Presumably such cells eventually rupture and discharge their contents into either the body cavity or the lumen of the tubule, but this process is not shown in any section yet examined.

RELATION OF THE *RICKETTSIA* BODIES TO THE THREAD FORMS.

Under dark ground illumination the thread forms have been observed to evacuate granules which, judging by their size, are similar to the darkly stained bodies seen within the thread forms in smear preparations.

Circumstantial evidence suggests that these granules are the same as the *Rickettsia* bodies which in the first instance attracted our attention and that in bugs which have fed these bodies are largely derived from disruption of thread forms.

PROBLEM OF THE INFECTION OF THE EGGS.

In addition to their development in certain cells of the alimentary system, thread forms have been found in the blood of the bug taken with a fine capillary pipette from the cut stump of one of the legs of a female and heavy infection of certain cells of the organ of Berlese has been observed in an apparently virgin female (no spermatozoa were observed either in this organ or in a smear of the ovaries which contained no developed eggs). The smear of the ovaries of this female also showed infection, but it was uncertain whether the egg cells were themselves infected. In the case of this apparently virgin female the form found in the sexual organs was chiefly the lanceolate form.

The testes of the males are also infected in some cases but no signs of the parasite were found in smears of the accessory glands of the same insects. As already stated there is no evidence that the organism is motile. It seems possible that the eggs may be infected by the spermatozoa but attempts to obtain evidence from teased preparations that the spermatozoa were infected gave only negative results.

GENERAL CHARACTER OF THE INFECTION.

Heavy infection with this parasite must be very general, if not universal, among bed bugs. The stock of bugs in which the parasite was first noticed originated from a few specimens taken from an old Essex cottage and as the insects had been confined in a small box and inbred for many generations prior to their use for the trench fever experiments above-mentioned, it is not surprising that almost every bug examined showed one or other of the forms of the parasite. Another race was obtained from an old house situated in Paddington and smears were made from bugs of this stock before breeding from it. Insects of this stock were also found to be generally infected. A further supply was obtained from animal cages in a London laboratory and examined without giving them any chance of further feeding, 19 out of 20 showing the presence of the parasite.

Bugs from two different sources in Warsaw were examined within a day or two of capture and all these were found to be infected.

DEVELOPMENTAL CYCLE OF THE PARASITE.

The scheme set out below is merely tentative, all that is claimed for it is that it is a reasonable explanation of the observed facts and the excuse is offered that by linking up these facts a clearer picture can be conveyed than by the mere recital of the detached details. In the absence of any success in cultivation apart from the host, it is of course only possible to assume a connection between some of the forms in the order in which they are stated.

Starting with the eggs within the ovary, it seems most probable (a) that they become infected at the time of fertilisation with the *Rickettsia* form (Plate II, fig. 7), (b) that simple multiplication is followed by some of the first generation developing through a bacillary stage into threads while others continue simple multiplication *pari passu* with the presentation of suitable conditions due to the development of the embryo, (c) that owing to the massive granule infection of the egg material at this stage, cells of practically every organ of the growing embryo tend to become involved, but only in a certain number are the conditions necessary for intracellular multiplication afforded. In these, clusters of the minute *Rickettsia* forms develop, rapidly changing in favourable situations into the red-staining lanceolate forms through the development of an outer covering or envelope. These enlarge in due course into the long bacillary forms which in their turn release the darkly staining minute *Rickettsia* bodies. The large cells of the Malpighian tubules and their free unencumbered position allow of a much more massive infection than other situations; it is also possible that they afford more stable conditions during the moulting periods and bring the organisms into close proximity to the sexual glands when these are developed, thus increasing the chances of the latter becoming infected. In the course of the very numerous dissections which it has been necessary to make in the progress of this work, it has been noticed how frequently the enlarged cells occur in the distal third of the tubules.

SUGGESTED NAME FOR THE PARASITE.

The organisms known as *Rickettsia* are at present very incompletely described and only recognisable by their more superficial characters, such as morphology and localisation.

On such evidence it is not possible to decide with any certainty how near or remote the phylogenetic relationship of the members of the group to one another may really be, and the present writers therefore consider that *Rickettsia* should not be accepted as a generic name in the strict sense of the term to cover all the organisms referred to in their opening paragraphs. The smaller group comprising only those concerned with the three mammalian (human) diseases typhus, trench, and Rocky Mountain spotted fevers, would seem to form a more convenient unit.

Nevertheless, since the Rocky Mountain spotted fever organism has been

put into a new genus by Wolbach and on the other hand the form in the sheep "ked" which is not believed, however, to infect the sheep has been called *Rickettsia melophagi* it will be useful to retain *Rickettsia* as a group name for all in the present state of our knowledge. It is in this latter sense that we use it in tentatively naming the above-described parasite of the bed-bug *Rickettsia lectularia*.

POSTSCRIPT.

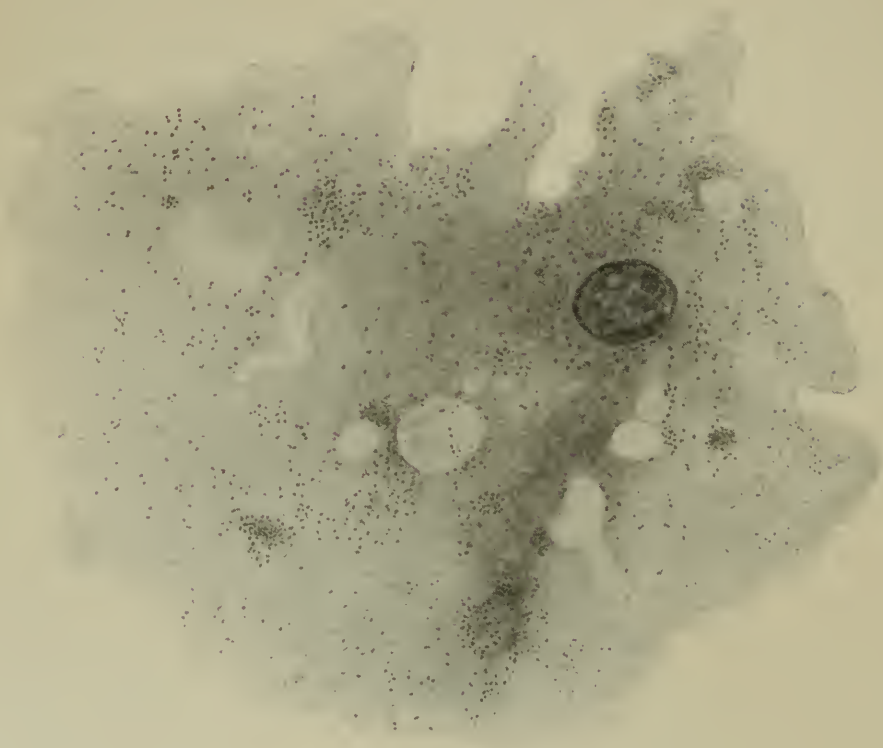
A recent examination of the few specimens of *Cimex hirundinis* which comprise all the bugs of the genus *Cimex*, apart from *C. lectularius* we have been able to obtain up to the present, shows that this species is also parasitised in some of the same organs which are infected in *C. lectularius*. As both the ovaries and testes are involved it is very probable that this will also prove to be an hereditary infection. The organism is, however, much more bacillary in appearance, considerably larger in size and has different staining reactions; in spite of several similarities it is not possible in the present limited state of our knowledge to decide how nearly, if at all, it may be related to the organism described above. We hope to obtain further material to continue our research and should any of our readers be able to obtain living specimens of any of the species within the genus *Cimex*, other than *lectularius*, we shall be very grateful to them if they will send us some.

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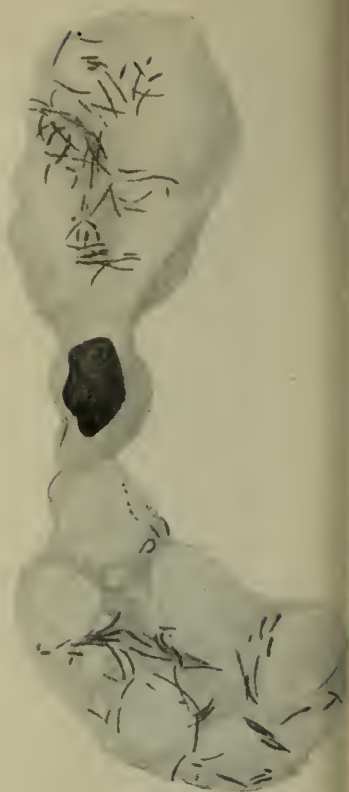
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DESCRIPTION OF PLATE II.

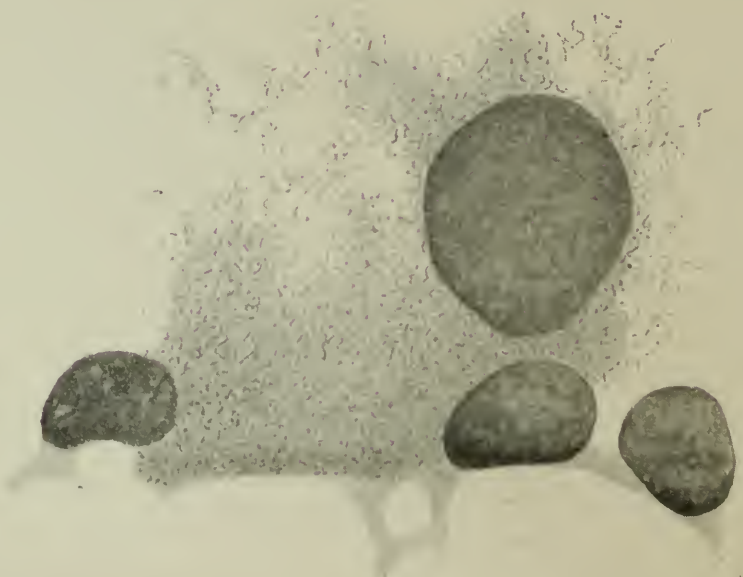
- Fig. 1. Minute lanceolate forms issuing from ruptured cell of Malpighian tube ($\times 1000$).
Fig. 2. Bacterial forms in fragment of gut wall ($\times 1300$).
Fig. 3. A large cluster of lanceolate forms and developing bacterial forms issuing from ruptured cells of ovary ($\times 1000$).
Fig. 4. Minute lanceolate forms issuing from ruptured cell of organ of Berlese ($\times 1300$).
Fig. 5. Thread forms issuing from ruptured end of Malpighian tube ($\times 1000$).
Fig. 6. Section through Malpighian tube showing mass of thread forms in infected cell ($\times 1000$).
Fig. 7. Rickettsia and bacillary forms from smears of two eggs. The Rickettsia forms are more heavily stained than is usual in smears of eggs ($\times 1300$).
Fig. 8. Smear of Malpighian tube showing Rickettsia and thread forms ($\times 1300$).
Fig. 9. A range of forms selected from smears of various organs ($\times 1300$).



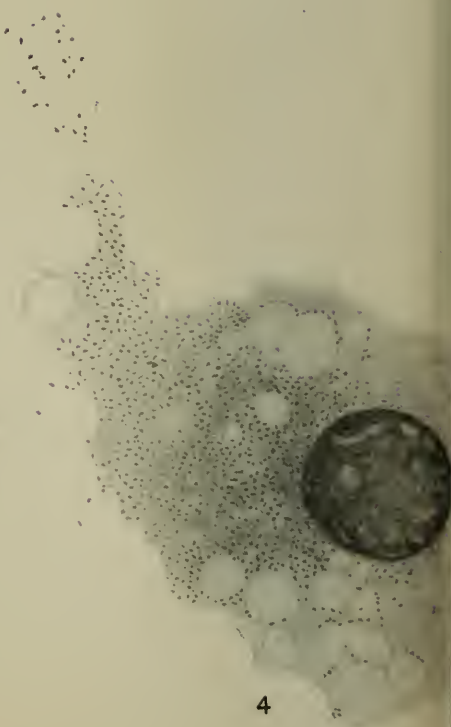
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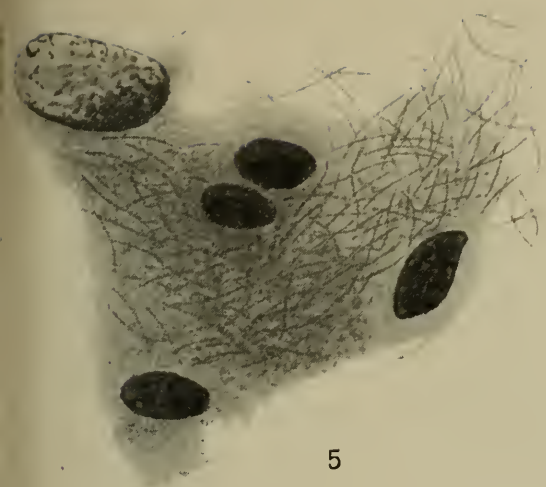
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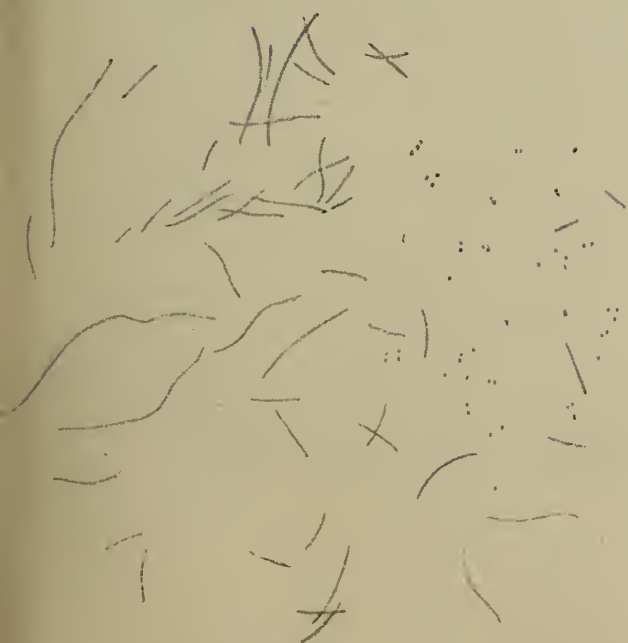
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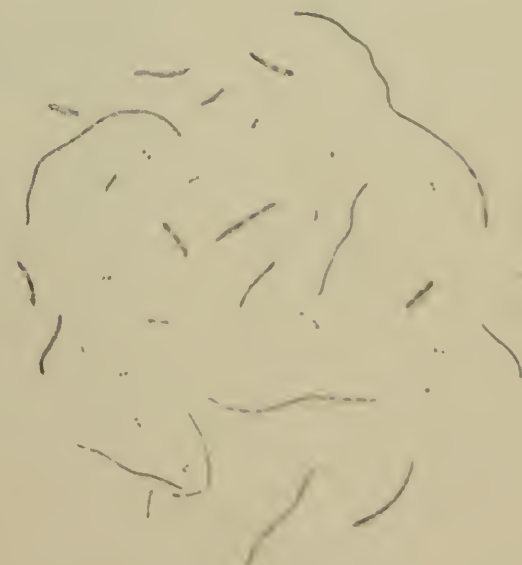
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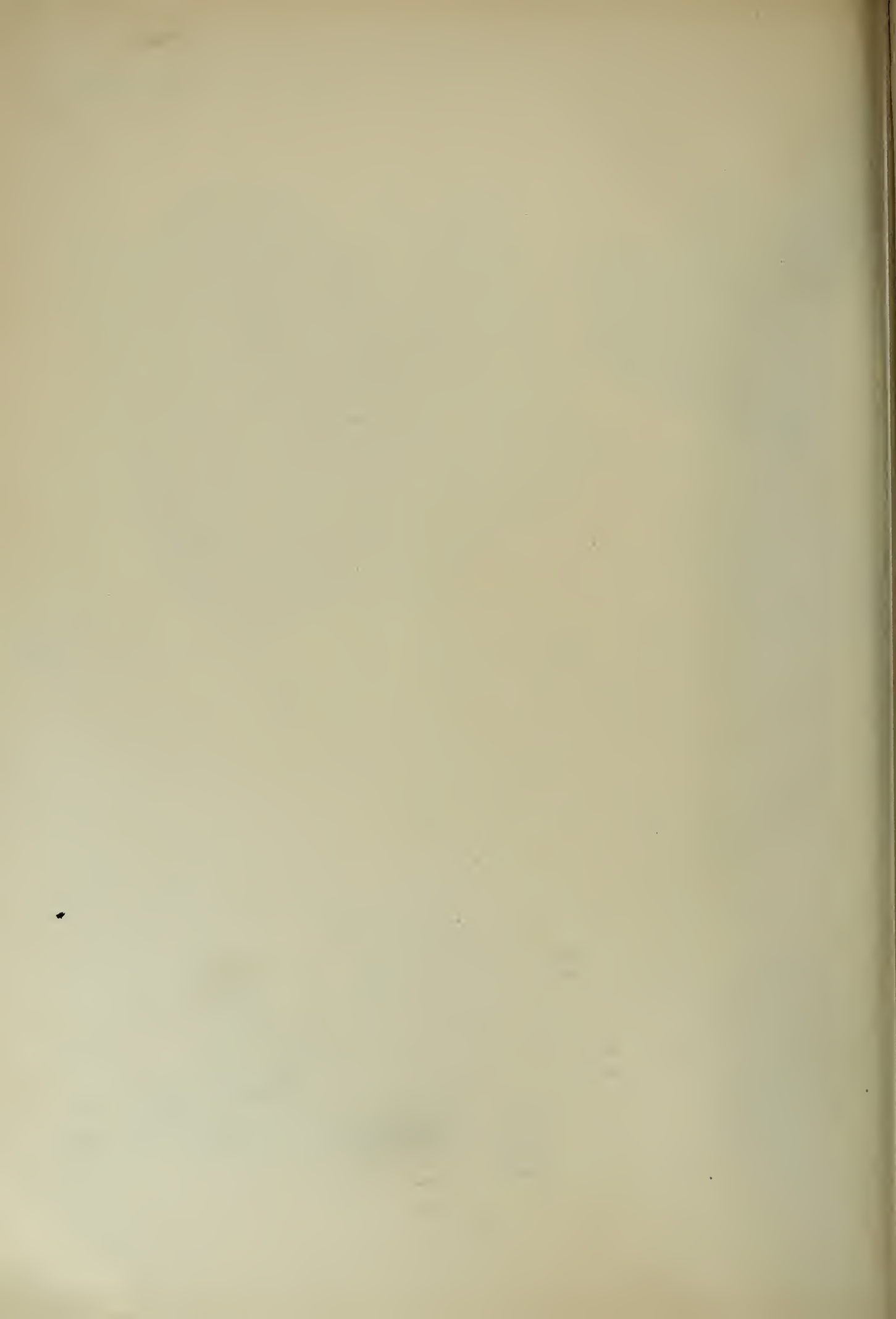
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9



ON THE PROBABLE IDENTITY OF RICKETTSIA PEDICULI WITH RICKETTSIA QUINTANA.

BY

A. BACOT, F.E.S.,

ENTOMOLOGIST TO THE LISTER INSTITUTE OF PREVENTIVE
MEDICINE.

MUNK and da Rocha-Lima (1917)¹ and Brumpt (1918)² found rickettsia bodies in lice taken from "healthy persons"; the two former writers named this supposed distinct species found by them *Rickettsia pediculi*, although they stated that it was indistinguishable morphologically and in its distribution in the louse from *R. quintana*, which they found associated with lice that had fed on trench fever patients. They infer that there is a form of rickettsia, infecting the gut of lice, which has no relation to trench or typhus fever. If this is correct, the discovery of these organisms in the lumen of the gut has no necessary significance. My colleague, Dr. Arkwright, and myself have, however, examined numbers of lice taken from civilian clothing in London and have never seen rickettsia bodies except when the insects have fed upon patients who have suffered from trench fever, and it is accordingly conceivable that this disease may have existed unrecognized among the population from which Munk and da Rocha-Lima and Brumpt obtained their lice.

The following account of a case of trench fever occurring in a region where the disease was supposed to be non-existent, in which the rickettsia bodies appeared in lice shortly after the insects were fed upon the patient, is of interest in this respect.

In April, 1920, I experienced while in Warsaw a febrile attack which exhibited the clinical signs of trench fever. The clinical evidence was, moreover, supported by the development of rickettsia bodies in the lice which I took to Poland with me. All the details of the case were under the observation of Professor Wolbach of Harvard, and Professor J. L. Todd³ of McGill, who agreed (1920) that the symptoms were those of trench fever.

On February 18th I joined Professors Wolbach and Todd in Paris in the capacity of Entomologist to the Typhus

I. THE ACTION OF SODIUM HYDROXIDE UPON COAGULATION OF FIBRINOGEN.

BY JOHN OGLETHORPE WAKELIN BARRATT.

Beit Memorial Research Fellow.

From the Lister Institute.

(Received October 28th, 1920.)

THE well-known action of sodium hydroxide in retarding coagulation of fibrinogen in presence of thrombin has been variously interpreted. J. Mellanby [1909] regards this retardation as due to the solution of the newly formed fibrin by the alkali and to a destruction of the ferment. According to W. H. Howell [1916] the effect of addition of alkali is to prevent the formation of fibrin fibrils, though the formation of fibrin aggregates, to which this author regards gelatinisation of fibrinogen as due, may still occur.

With a view of further investigating the action of alkali upon the coagulation of liquids containing fibrinogen and thrombin the present work was undertaken.

It is obvious that if the action of alkali is exerted exclusively upon fibrinogen or is directed solely to thrombin, it would be easy to determine the nature of this action by a comparison of (1) the effect upon coagulation of adding increasing amounts of alkali to plasma or fibrinogen solution with (2) the course of coagulation in a series of dilutions of (a) fibrinogen, to each of which the same amount of thrombin has been added, and (b) thrombin, to each of which the same amount of plasma has been added.

The effect of dilution of fibrinogen is shown in Table I, that of dilution of thrombin in Table II and the action of alkali in Table III. In these experiments fibrinogen, which cannot at present be prepared in even an approximately pure condition, was represented by citrated human plasma: it is well known that when fibrinogen is precipitated from plasma by dilution and acidification relatively considerable amounts of albumin and globulin are carried down in the precipitate [J. Mellanby, 1905]. As source of thrombin the venom of *Echis carinatus* [Barratt, 1913], dissolved in citrated 0.85 % sodium chloride solution, was employed¹. The volume of citrated plasma used (undiluted or diluted) was in each experiment 0.050 cc.: to this was added 0.0026 cc. of citrated sodium hydroxide solution of suitable concentration (Table III) or of citrated 0.85 % sodium chloride solution (Tables I

¹ For a supply of this venom I am indebted to the kindness of Dr C. J. Martin, Director of the Lister Institute.

Table I.

To citrated human plasma, undiluted and in the dilutions given below, venom was added in amount required to produce a dilution of 1 in 3,000,000. The time of first appearance of fibrils is calculated from the equation on p. 6.

Plasma %	Time of first appearance of fibrils (min.)		Dimensions (approximate) and aspect of fibrils
	observed	calculated	
100	24	24	.25 μ thick; 50 μ long; numerous; meshes wide
90	24	24	
80	23	24	.25 μ thick; 50 μ long; numerous; meshes wide
70	50	55	
60	110	99	.25 μ thick; 50 μ long; numerous; meshes wide
50	140	151	.25 μ thick; 50 μ long; less numerous; meshes fairly wide
40	191	215	.2 μ thick; 40 μ long; less numerous; meshes less wide
30	360	297	.1 μ thick; 7 μ long; scanty; barely visible
20	no fibrils seen	414	

and II); finally 0.0026 cc. of citrated venom was added, of such concentration as would furnish, after admixture, the titre indicated in the tables. Immediately after mixing, a few cubic millimetres were placed on a microscope slide within an area ringed with vaseline and examined on a dark ground, illuminated by an arc light. The temperature of experiment was 16–18°.

On comparing Tables I and II it will be observed that the effect of dilution of fibrinogen (the amount of thrombin added being the same in each experiment) or of dilution of thrombin (the amount of fibrinogen present being the same in each experiment) is to cause (1) delay in the appearance of fibrin fibrils and (2) diminution in their number. Here, however, the resemblance

Table II.

To citrated human plasma (undiluted) venom was added in amount required to produce the dilutions given below. The times of first appearance of fibrils are calculated on the assumption that concentration of venom \times time of first appearance of fibrils = 8.3×10^{-6} .

Venom	Time of first appearance of fibrils (min.)		Dimensions (approximate) and aspect of fibrils
	observed	calculated	
1 : 600,000	5	5	.11 μ thick; 5 μ long; fine; very numerous; meshes fine
1 : 1,000,000	8	8	
1 : 1,300,000	11.5	11	.2 μ thick; 15 μ long; numerous; meshes less fine
1 : 1,500,000	10.5	12.5	
1 : 2,000,000	19	16.5	.25 μ thick; 40 μ long; numerous; meshes wide
1 : 3,000,000	26	25	.25 μ thick; 50 μ long; numerous; meshes wide
1 : 6,000,000	47	50	.3 μ thick; 60 μ long; fairly numerous; meshes larger
1 : 12,000,000	175	100	.35 μ thick; 80 μ long; less numerous; meshes very wide
1 : 24,000,000	350	200	.5 μ thick; 150 μ long; scanty; coarse

in the two cases ceases: with increasing dilution of fibrinogen the fibrils become short and fine, and ultimately are barely visible (Table I); with increasing dilution of thrombin, on the other hand, the fibrils become long

and coarse (Table II). The effect of adding alkali (Table III) is seen to be identical with that of diluting fibrinogen: this is very strikingly seen when the penultimate experiment in Table III is compared with the penultimate experiment in Table I and with the last experiment in Table II. It follows, therefore, that the action of alkali is exerted upon fibrinogen: no recognisable effect upon thrombin is observable.

The experiments in Table I, when plotted with concentration of plasma as ordinates and time of first appearance of fibrin fibrils as abscissae are found to lie at first upon a vertical line and then upon a curve, as is shown in Fig. 1. Fibrinogen in solution has been shown to be diphasic [Barratt, 1920], the time of first appearance of visible fibrils (made up almost entirely of the period occupied by the growth of the primitive fibrils, which thereby pass from invisibility into the range of ultramicroscopic vision, this growth occurring at the expense of fibrinogen derived from the continuous or dilute phase) being unaltered by dilution until the disperse (concentrated) phase has nearly completely passed into solution (dilute phase), after which further dilution causes a lowering of the concentration of the dilute phase and as a

Table III.

To citrated human plasma (undiluted) sodium hydroxide was added in quantities corresponding to the concentrations given below. Venom was then added in amount required to produce a dilution of 1 in 3,000,000.

Exp.	NaOH	Fibrils first seen at end of	Dimensions (approximate) and aspect of fibrils
1	0	24 min.	$\cdot 25\mu$ thick; 50μ long; numerous; meshes wide
2	1/120 <i>N</i>	23 "	
3	1.3/120 <i>N</i>	23 "	$\cdot 2\mu$ thick; 20μ long; numerous; meshes small
4	1.6/120 <i>N</i>	24 "	
5	1.9/120 <i>N</i>	50 "	$\cdot 2\mu$ thick; 20μ long; very numerous; meshes very small
6	2.2/120 <i>N</i>	54 "	
7	2.5/120 <i>N</i>	63 "	$\cdot 15\mu$ thick; 10μ long; very numerous; meshes very small
8	2.8/120 <i>N</i>	150 "	
9	3.1/120 <i>N</i>	173 "	$\cdot 1\mu$ thick; 5μ long; very numerous; barely visible
10	3.4/120 <i>N</i>	285 "	
11	3.7/120 <i>N</i>	345 "	$\cdot 1\mu$ thick; 5μ long; less numerous; barely visible
12	4/120 <i>N</i>	no fibrils seen	

result retardation of the growth of primitive fibrils (exhibited by a lengthening of the period of first appearance of visible fibrils). The curve, it may be observed, can be calculated from the equation

$$-lc = kt + a$$

where c = concentration of fibrinogen, $t + 24$ = time of first appearance of fibrils and k and a are constants, represented by 0.0035 and -4.357 respectively.

On reference to Tables I and III it will be seen that in the earlier experiments of each series the period in which visible fibrils first appear is the same,

namely 23 to 24 minutes: only in the later experiments is a retardation observable. This behaviour indicates that the result of adding alkali to plasma is to cause disappearance of fibrinogen, the disperse (concentrated) phase in consequence becoming reduced in amount but the continuous (dilute) phase not being affected until the former phase has nearly completely disappeared, and is therefore no longer able on further dilution to maintain the saturation concentration of the continuous phase, the process occurring being in this respect comparable in its effect to simple dilution of fibrinogen. The concentration of fibrinogen corresponding to the periods given in Table III,

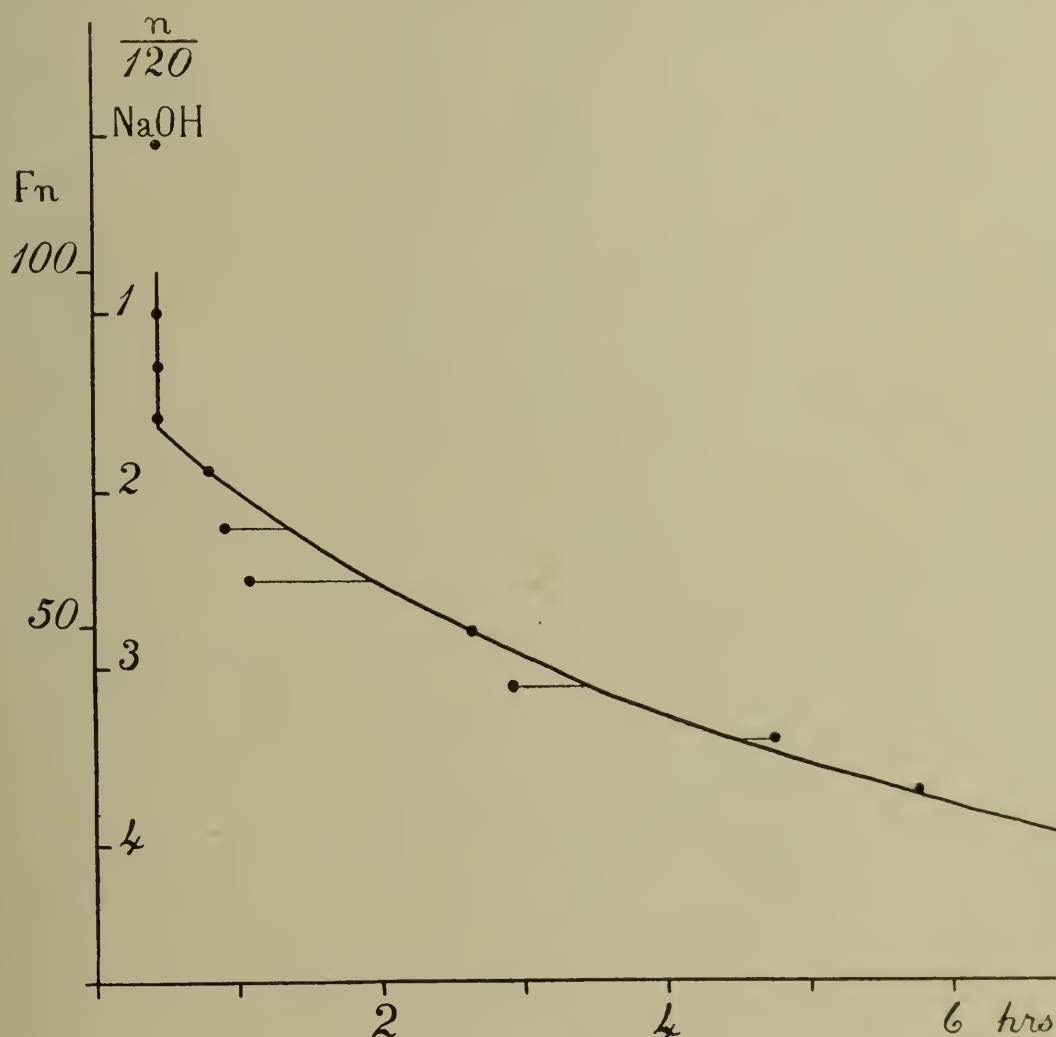


Fig. 1. Graphic record of experiments given in Tables I and III. Ordinates indicate (1) on the left concentration of fibrinogen, that present in undiluted citrated human plasma being represented by 100, and (2) on the right titre of sodium hydroxide; abscissae show time of first appearance of fibrin fibrils. On the curve, which represents the experiments in Table I, the estimated disappearance of fibrinogen on addition of alkali in Tables III and IV is exhibited.

col. 3, may be determined graphically from the curve in Fig. 1 (or by calculation with the aid of the equation on p. 6). The figures so obtained are shown in Table IV, col. 2. If the average amount of fibrinogen disappearing for each increment of alkali is determined (namely that contained in approxi-

mately 7.5 % of plasma for each increment represented by 0.3/120 *N* NaOH) the percentages of fibrinogen given in the third column of Table IV are obtained: these are indicated in Fig. 2, the amount of fibrinogen present in undiluted plasma being represented by 100. (It will be seen from Fig. 2 that no action of alkali on fibrinogen is observable until an amount represented by 0.74/120 *N* has been added.)

Table IV.

Disappearance of fibrinogen on addition of sodium hydroxide in experiments recorded in Table III.

Exp.	Fibrinogen	
	observed	calculated
1	100	100
2	<100	94.5
3	<100	87
4	<100	79.5
5	72	72
6	71	64.5
7	68	57
8	50	49.5
9	47	42
10	32	34.5
11	27	27
12	—	20

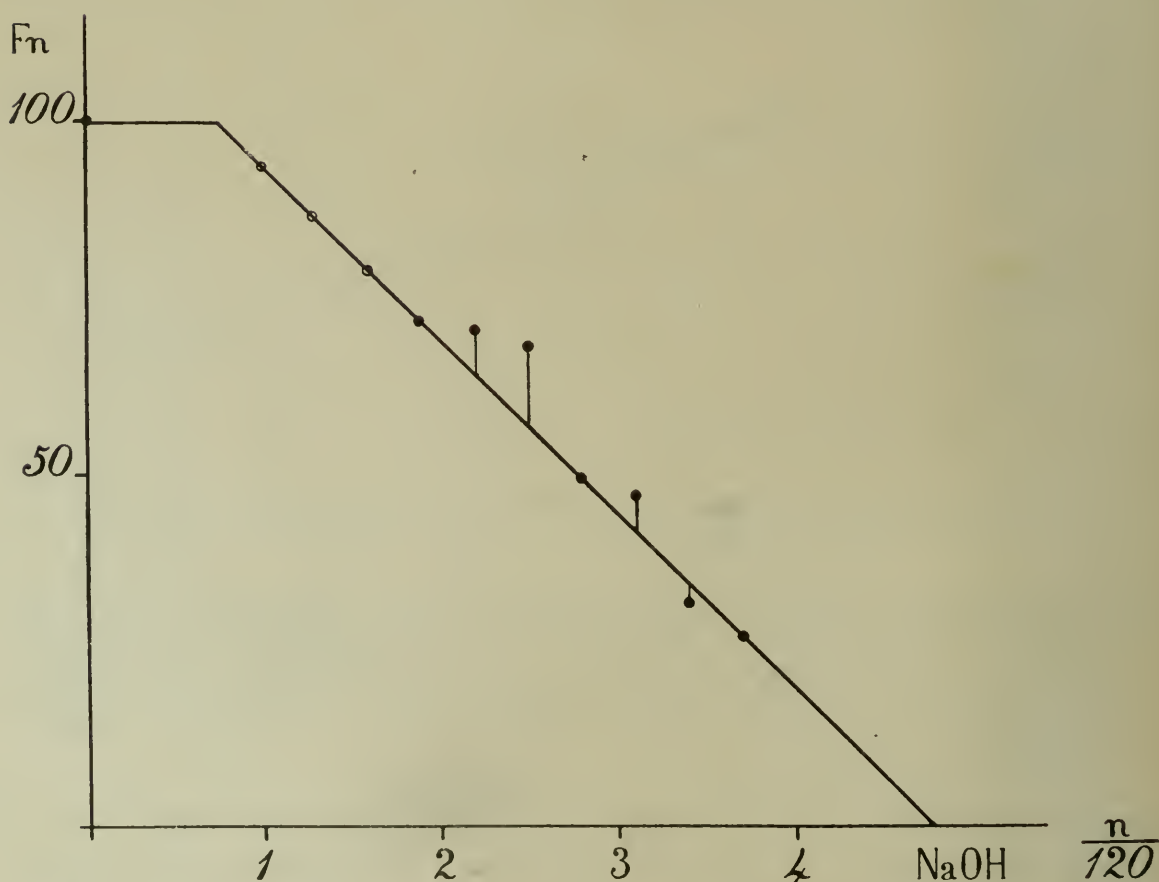


Fig. 2. The relation between amounts of sodium hydroxide added in the experiments given in Table III and corresponding estimated diminution of fibrinogen (Table IV).

The variations from a simple proportionality between addition of alkali and disappearance of fibrinogen in the individual experiments recorded in Tables III and IV are most conveniently exhibited graphically, as in Fig. 2. These variations lie within the error of experiment inseparable from the circumstance that on the one hand the volume in which the thrombin and alkali added is contained must be kept very small relatively to that of the plasma (fibrinogen) employed, in order that the concentration of the latter may not be lowered thereby in any marked degree (in these experiments the respective volumes of (1) plasma and (2) thrombin or alkali solutions employed were 19 : 1), while on the other hand owing to the necessity of rapid working the amounts of fluid used must be small. Although the fibrinogen used for experiment was contained in plasma and the experiments given in Tables III and IV indicate, as already noted, the occurrence of a subsidiary reaction, nevertheless the results obtained suggest that the relation between alkali and fibrinogen is stoichiometric. As is well known fibrinogen is not destroyed by alkali: on addition of carbonic acid to alkali plasma the fibrinogen which has disappeared reappears and clotting in presence of thrombin is now readily obtained. The action of fibrinogen in respect of alkali thus appears to be comparable to that of an extremely weak organic acid.

With the increasing dilution of fibrinogen exhibited in Table I, the fibrin fibrils become gradually finer until ultimately they can no longer be recognised. When this stage is reached there is, however, still evidence of the existence of fibrils, though no longer capable of demonstration by ultramicroscopic illumination, for soft clot continues to be formed *in vitro*. It must therefore be inferred that in such apparently structureless clot amicroscopic fibrils are present, though, owing to the dilution of the continuous phase, no longer able to increase sufficiently in size, by further addition of fibrinogen from this phase, to become visible. In support of this inference, reference may be made to the circumstance that under suitable conditions of experiment the formation of structureless clot precedes visible fibril formation: thus when the dilution of fibrinogen is just insufficient to lead to the non-appearance of fibrin fibrils, "setting" *in vitro* may be observed to precede the appearance of visible fibrils on the microscope slide. It has already been shown [Barratt, 1920] that the formation of primitive (amicroscopic) fibrils precedes by a relatively considerable time the appearance of visible fibrils.

When alkali is added to citrated human plasma the same phenomenon is observable with much greater facility, owing to the circumstance that the volume of solution of alkali added may be made very small compared with that of the plasma employed, and thus the diminution in number of fibrils dependent upon simple dilution avoided, with the result that greater firmness of clot is secured. If sufficient alkali is added to plasma to cause disappearance of visible fibrils, a structureless gel of soft consistence is still obtainable: as more alkali is added gel formation becomes uncertain and ultimately ceases. Thus in a series of experiments of the type shown in Table III, in which the

concentration of venom was 1 in 600,000, no visible fibrils were observed after the fifth experiment: in experiments 6 to 9 "setting" *in vitro* occurred in periods ranging from 30 minutes to 3 hours; in the remaining experiments "setting" was imperfect or did not take place.

The appearance of structureless gel after addition of alkali was noted by Howell [1916], who moreover obtained structureless gel from plasma on dilution and on drying.

Addition of alkali to plasma in amount sufficient to delay fibril formation does not at first affect phenolphthalein. No marked red coloration could be observed until fibrils were no longer recognisable and gel formation absent: in other words, until the amount of sodium hydroxide added was as much as would, if added to a volume of distilled water equal to that of the fibrinogen solution employed, give to the latter a concentration of alkali represented by $1/30 N$.

SUMMARY.

(1) Sodium hydroxide, added in increasing amounts to citrated plasma, causes the gradual disappearance of fibrinogen from the plasma: the fibrin fibrils observable in the gel forming on addition of thrombin diminish in size and number, and ultimately pass out of the range of ultramicroscopic visibility; when this stage is reached gel formation may be still observable, but the gel obtained is structureless under microscopic illumination, being made up of amicroscopic fibrils; with still further disappearance of fibrinogen gel formation ceases.

(2) No evidence of any action of alkali upon thrombin was obtained.

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XLVII. THE HEAT INACTIVATION OF DIPHTHERIA ANTITOXIN.

By ANNIE HOMER.

(Received May 18th, 1920.)

AMONGST serum workers there is an impression that the addition of from 1·5 to 2 % of sodium chloride to antitoxic plasma, prior to its being heated, guards against the undue inactivation of the antitoxin, and it is generally accepted that the protective influence of the sodium chloride in this respect is still more marked during the heating of cresylised plasma or serum.

In order to furnish definite evidence on this point a study has been made of the percentage inactivation of antitoxin during the heating of:

(A) Oxalated antitoxic plasma,

(B) Plasma (A) to which an addition of 1·5 % of solid sodium chloride was made,

(C) Oxalated antitoxic plasma containing 0·30 % of cresylic acid,

(D) Plasma (C) to which an addition of 1·5 % of solid sodium chloride was made,

for stated periods of time at temperatures ranging from 58° to 72°.

Separate volumes of the respective plasmas (A), (B), (C) and (D), each of 25 cc., contained in 30 cc. sealed ampoules, were heated for definite periods of time in water baths regulated to the specified temperatures by means of thermostats.

Determinations were made of the antitoxic values of the respective liquids before and after heating, and, from the results thereby obtained, was calculated the percentage inactivation induced during the heating period.

The data thus furnished from the study of the plasmas (A) and (B), and of (C) and (D), have been included in Tables I and II respectively.

It will be seen that the rate of inactivation rapidly increases with the rise in temperature. Thus (Table I), while the plasma can be kept at 58° for ten hours without suffering any appreciable diminution of its antitoxic value, there is a 10 % loss shown after three hours' heating at 60·5°, or after two hours' heating at 61·5°, or after one hour's heating at 63°. With the progressive rise in temperature the proportional loss of antitoxic value in a given time rapidly increases until at 72° there is a 60 % inactivation of the antitoxin within ten minutes.

A comparison of the results given in Tables I and II shows that the loss of antitoxin during the heating of cresylised plasma is greater than that resulting from the similar treatment of noncresylised plasma. But, contrary to the ideas current amongst serum workers, the evidence obtained in this investigation indicates that the addition of sodium chloride does not appreciably lessen the heat inactivation of antitoxin either in cresylised or in noncresylised plasma.

Further information as regards the heat-instability of antitoxin was obtained from a similar study of the inactivation of antitoxin in solutions containing only those protein fractions with which the antitoxin is associated in plasma.

The salt-soluble globulin fraction of an unheated antidiphtheritic plasma (potency 450 units per cc. and protein content 7.48 %) was separated and dialysed in the usual way. The residue from dialysis was diluted with distilled water so as to reduce the protein content of the liquid to 7 %.

This solution of antitoxin and its associated proteins, freed in this way from albumin, euglobulin and the other organic and inorganic constituents of the plasma, was submitted to the same treatment as that described above for the cresylised and noncresylised plasmas, (A), (B), (C) and (D).

The results recorded in Table III indicate that, as was anticipated, antitoxin shows a greater stability to heat when in aqueous solution associated with its own particular proteins than when present in the more complex medium of the plasma.

While studying the effect of heat on the inactivation of antitoxin I also made observations on the accompanying increased precipitability of the serum proteins. From the data, thus obtained, was calculated the percentage denaturation of the serum proteins, the results for the respective liquids being also included in Tables I, II and III.

In all cases it was noticed that, during the heating of those sera or plasmas which contained from 1.5 to 2 % of sodium chloride or cresylic acid and phenol in amounts up to 0.6 %, there was an apparently greater conversion of soluble into insoluble protein than occurred during the heating of noncresylised plasma. However, by means of the refractometer, it was ascertained that the presence of sodium chloride or of cresylic acid and phenol in the plasma had not affected the extent of the heat-denaturation of the proteins, these substances had merely stimulated the coagulation of the particles of denaturated proteins into aggregates of sufficient size to be more readily discerned by the eye.

From the results recorded both here and in my previous papers it has been found that the extent of the denaturation of the proteins is a function of:

- (a) the temperature at which the heating was conducted;
- (b) the reaction of the plasma;
- and (c) the duration of the heating of the plasma.

Table I.

Showing the extent of the denaturation of the serum proteins and the inactivation of the antitoxin during the heating of oxalated antidiphtheritic plasma (A) and of the same plasma to which has been added 1.5 % of sodium chloride (B).

Potency of the unheated plasma = 375 units per cc.

Protein-content of the plasma = 8.45 %

P_H of the unheated plasma, 7.8.

Temperature °C.	Duration of the heating	Plasma (A)		Plasma containing 1.5 % NaCl (B)	
		Percentage denaturation of the serum proteins	Percentage inactivation of the antitoxin	Percentage denaturation of the serum proteins	Percentage inactivation of antitoxin
58	4.5 hours	30.3	nil	30.5	nil
"	10 "	31.0	"	31.2	"
60.5	1 hour	13.5	"	14.0	"
"	2 hours	40.0	"	41.0	"
"	3 "	42.0	10	41.5	less than 10
61.5-62	1.5 "	40.0	—	40.0	—
"	2 "	50.0	8	49.0	less than 8
"	3 "	55.0	slightly greater than 13	55.0	about 20
"	4 "	56.5		57.5	
63	1 hour	52.0	10	53.5	10
"	2 hours	64.0	slightly less than 27	66.0	25
"	3 "	72.0	—	70.0	—
65	10 minutes	40.8	—	42.5	—
"	20 "	57.0	about 27	58.5	about 27
"	30 "	65.0	about 33	66.0	about 33
"	1 hour	practically solid	—	practically solid	—
66	10 minutes	44.5	about 13	45.5	about 10
"	20 "	60.0	slightly less than 33	62.0	slightly less than 33
67.5	10 "	62.5	20	63.5	20
"	20 "	69.0	36	71.0	33
68.5-69	5 "	56.5	—	57.0	—
"	10 "	62.0	33	61.0	33
"	20 "	72.0	slightly less than 46	72.0	slightly less than 46
70	5 "	65.0	slightly less than 33	65.0	33
"	10 "	75.0	53	75.0	slightly greater than 46
"	15 "	almost solid	—	practically solid	—
72	5 "	80.0	60	80.0	60
"	10 "	practically solid	—	practically solid	—

Table II.

Showing the extent of the denaturation of the serum proteins and the inactivation of antitoxin induced during the heating of antidiphtheritic plasma containing 0.30 % of cresylic acid (C), and of the same cresylised plasma to which 1.5 % of sodium chloride has been added (D).

Potency of the original plasma = 375 units per cc.

P_H of the original plasma, 7.4.

Protein-content of the plasma = 8.45 %.

Temperature °C.	Duration of the heating	Cresylised plasma (C)		Cresylised plasma containing 1.5 % of NaCl (D)	
		Percentage denaturation of the serum proteins	Percentage inactivation of the antitoxin	Percentage denaturation of the serum proteins	Percentage inactivation of the antitoxin
58	4 hours	25.0	nil	25.4	nil
"	8 "	26.0	"	26.0	"
60	1 hour	41.6	negligible	40.6	negligible
"	2 "	45.5	10	45.4	10
"	3 "	48.5	17	48.3	17
62	1 "	45.0	17	47.2	13
"	2 hours	55.0	—	57.5	24
"	3 "	57.0	—	57.5	—
63	1 hour	47.2	17	50.0	17
"	2 hours	64.0	—	65.0	—
"	3 "	70.0	—	practically solid	—
64	30 minutes	53.0	15	52.0	17
"	1 hour	63.5	—	66.0	—
"	2 hours	practically solid	—	practically solid	—
65	20 minutes	53.0	40	52.0	40
"	30 "	65.0	—	64.0	—

The extent of the inactivation of the antitoxin seems to be regulated by:

(a) the temperature at which the heating is conducted;

and (b) the time of heating.

The time of heating required to produce the maximum value for the denaturation of the proteins became shorter as the temperature was raised. In this respect the behaviour of antitoxin and of the serum proteins is somewhat analogous. However the heat-inactivation of the antitoxin seems to be mainly a function of the temperature and independent of the extent of the accompanying heat-denaturation of the serum proteins, for, by suitable adjustments, the heat-denaturation of the proteins at a given temperature can be rendered complete without any corresponding change in the coefficient for the heat-inactivation of the antitoxin at that temperature.

These observations lead to considerations as to whether antitoxins should be regarded as separate entities rather than as structural modifications of the serum proteins induced during the process of immunisation. In this connection, it had been my intention to plot curves for the time taken, at each degree of temperature, to reduce the antitoxic value of the liquids to half

Table III.

Showing the extent of the heat-denaturation of pseudo-globulin and of the inactivation of antitoxin in solutions which have been heated at temperatures between 58° and 72°.

P_H of the solution of pseudo-globulin, 7.0.

Potency = 450 units per cc.; protein-content, 7.48 %.

Temperature °C.	Duration of the heating	Percentage denaturation of the pseudo-globulin	Percentage inactivation of antitoxin
58	4 hours	25.0	negligible
"	10 "	26.0	"
61.5	1 hour	23.7	nil
"	2 hours	26.6	negligible
"	3 "	31.6	"
63	30 minutes	31.0	"
"	1 hour	40.0	"
"	2 hours	51.0	"
"	3 "	55.0	"
66	20 minutes	40.0	"
"	40 "	46.0	"
67.5	10 "	50.7	"
"	20 "	61.7	nearly 13
68.5	10 "	57.0	8
"	20 "	67.0	—
70.0	5 "	50	negligible
"	10 "	70	30
"	15 "	practically solid	—
72	8 "	90	greater than 50

value, and to make therefrom a comparison between the heat inactivation of antitoxin and that of certain enzymes. But, unfortunately, owing to the prolonged scarcity of experimental animals, I have been unable to carry my investigations further in this respect.

The work involved in this investigation was, in part, carried out during my war time appointment with the Lister Institute.

SUMMARY.

During the course of the investigation it has been found that:

(1) The addition of 1.5 to 2 % of sodium chloride to plasma, whether noncresylised or cresylised, prior to its being heated, in no way reduces the extent of the heat-inactivation of the antitoxin.

(2) The rate of the heat-inactivation of antitoxin is a function of the temperature at which the heating is conducted.

(3) The extent of the heat-inactivation of the antitoxin in an aqueous solution of the antitoxin bearing proteins is considerably less than that evidenced during the heating of antitoxic plasma.

N^o 8

THE CULTURAL DIAGNOSIS OF ENTERICA IN INOCULATED INDIVIDUALS

BY

J. C. G. LEDINGHAM, C.M.G., M.A., D.Sc.
M.B., CH.B. ABERD.

CHIEF BACTERIOLOGIST, LISTER INSTITUTE, LONDON.

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THE CULTURAL DIAGNOSIS OF ENTERICA IN INOCULATED INDIVIDUALS.

THERE appears to be an impression that preventive inoculation against enterica diminishes the chance of recovering the causative organism from the blood. Thus, in a recent paper by R. P. Garrow,¹ entitled "The Myth of 'Atypical' Enteric Fever," with the argument of which I am in sympathy, in substance at least if not in detail, the following statement occurs:—

"The difficulty of recovering enteric bacilli from the blood of inoculated soldiers is well known, even in the early stages of illnesses which clinically resemble enteric."

Is this a well-known fact? If so, I am not aware of any published data in its support. In bacteriological circles on the Western front during the late war it would appear that a similarly pessimistic view was held. Thus A. E. Webb-Johnson,² writing of his experiences at a stationary hospital in France, remarks:—

"With regard to inoculation there is another point, which is possibly one of the most important. The specific bacteria are not found in either blood, faeces, or urine with anything like the same frequency nor in such numbers as in the non-inoculated, since the microbes are killed more rapidly and effectually, owing to protective substances being in the body ready for the attack."

The fostering of such views gave, I imagine, the impetus to that concentration on agglutination tests which formed such a feature of enteric diagnosis in France. Largely on that basis of diagnosis cases of enterica have been analysed from the standpoint of clinical syndrome, and for some time it has been current teaching that inoculation profoundly modifies the type and severity of the attack in inoculated individuals. With this aspect of the case I am not immediately concerned, but I hold that any comparative analysis of clinical syndrome or of incidence and mortality statistics in inoculated and uninoculated individuals must, to be above all reproach, be founded on a culturally diagnosed series of cases.

Analysis of 373 Cases in American Army.

This has been done in a recently published and most valuable article by the late Major V. C. Vaughan,³ of the United States Army, whose conclusions are in agreement with those of Garrow (*loc. cit.*). This author submits with full bacteriological particulars a clinical analysis of 373 cases of enterica (mainly typhoid) which occurred among American troops in France, all of whom had been inoculated with the triple vaccine. For the purpose of his analysis the following proviso was laid down :—

“No case has been accepted for our statistical study as being undoubted typhoid or paratyphoid fever unless the causative organism has been isolated either from the urine or faeces, or preferably from the blood. I recognise that the finding of the typhoid or a paratyphoid bacillus in the faeces alone does not establish a diagnosis, but have assumed that this finding, together with the presence of characteristic clinical symptoms of the disease and with negative blood cultures, form sufficient evidence on which to base a diagnosis.”

A series of 270 cases in which *B. typhosus* was recovered from the blood or excreta (180 by blood culture) is taken for the main clinical study. His conclusions cannot be summarised here, and I need only say that the results of the analysis went to show that vaccination had not altered the essential characters of the disease.

“The most striking feature of the disease in the inoculated is its almost classical resemblance to the old typhoid as we knew it in the unvaccinated. Not only was this resemblance true on paper, but it was likewise true at the bedside.”

No convincing evidence was found that vaccination lessened the chance of finding the infecting organism, and in fact Vaughan's positive cultural results from blood, urine, and faeces reached remarkably high figures, as we shall see. Some of the bacteriological data are of great interest, and I propose to consider them in the light of certain data from the Mesopotamian laboratories now published for the first time. Of Vaughan's series of inoculated individuals 274 were submitted to blood culture (repeated if necessary) with a yield of 180 positive results—viz., 143 at first trial, 25 at second, 3 at third, and 9 at fourth. Blood culture was successful in the first week in 65·7 per cent. of trials, in the second in 54·7 per cent., in the third in 45·7 per cent., and in the fourth in 21·7 per cent. The urine was cultured in 109 cases with positive results in 33·9 per cent., and of 270 cases in which the stools were examined no fewer than 193 (70·2 per cent.) were successful. Simultaneous blood and faeces cultures were made on

150 cases in successive weeks of the disease, and the results showed that during the first week the chance of success by stool culture was about two-thirds of that obtainable by blood culture, whereas in the second week the chances were about even. In the third week stool culture gave a somewhat better chance. From the third week onwards the value of both methods decreased. To summarise, Vaughan's series of 373 cases of enterica in inoculated troops comprised 270 cases of culturally proved typhoid, 9 cases of culturally proved paratyphoid A, 23 of paratyphoid B, 12 classified as paratyphoid "indeterminate," and 59 cases of "clinical enteric" which did not yield to bacteriological inquiry. Vaughan's series of 373 cases must be regarded as a very specially observed and repeatedly investigated class, and it is not surprising that the yield of positive isolations reached the high figure of 84·1 per cent. all over.

Blood Culture Results from Mesopotamia.

With the varying conditions obtaining in a wide and scattered front such as we had in Mesopotamia, a figure such as this was not to be expected, but I am quite satisfied that even this figure may be attainable (and indeed was approached) in special circumstances where ward and laboratory act in partnership and all forces are concentrated on the diagnosis of enterica by isolation of the causative organism. I have summarised elsewhere⁴ some of the bacteriological statistics in connexion with enterica as met with in Mesopotamia, and I may simply repeat here that we had two classes only of notified enterica—viz., (1) confirmed enterica (isolation of T., A., or B.) and (2) clinical enteric group or E.G. Diagnosis by agglutination methods was not accepted officially and was not practised. During the period October, 1917, to December, 1918, of 1577 cases of notified enterica in British and Indian troops, 734, or 46·6 per cent., were confirmed by the laboratories (1039 British cases yielding 36·9 per cent. of confirmations and 535 Indian cases yielding 56·8 per cent. of confirmations). When all the circumstances are considered the figures are, in my opinion, highly creditable to the Mesopotamian bacteriologists and, as I have indicated, higher figures up to 70 or 80 per cent. were reached in certain hospitals dealing with small but thoroughly investigated series. Now, what facts emerge from a consideration of these cultural results in relation to previous inoculation? From my notes of the Mesopotamian laboratory returns I have abstracted the cases of successful blood culture for the period August, 1918,

to January, 1919 (six months). I find that of 139 cases diagnosed by blood culture 78 (viz., T. 22, A. 38, B. 18) were inoculated men, 28 were returned as not inoculated (T. 19, A. 5, B. 4), and 33 were set down as "no pay-book entry" (T. 15, A. 14, B. 4). Thus of the total at least 56.1 per cent. yielding positive blood cultures were definitely inoculated men; 35 had received T.A.B. vaccine in 1918, the year of their attack, 24 had received their last dose in 1917, 17 their last dose in 1916, and 2 in 1915 (probably typhoid vaccine only). There would, therefore, appear to be no justification for the assumption that in a mixed group of inoculated and uninoculated men it is the latter which are most likely to yield positive blood cultures.

Blood Culture and Period of Disease.

One other point may be usefully referred to here. Unsubstantiated statements have been made with regard to the duration of the bacteriæmia in typhoid and paratyphoid infections respectively as affecting the chance of successful blood culture. Accurate data on this matter can be obtained only by a very special investigation involving repeated blood culture in a series of culturally proved cases (by blood or otherwise), and so far such data are not forthcoming. It may, however, be of interest to bring forward certain figures connecting successful blood culture with period of disease in typhoid and paratyphoid infections respectively; I have notes of 102 cases of typhoid, 84 of paratyphoid A, and 26 of paratyphoid B. The results in 4-day periods are as follows:—

		No. of cases diagnosed of		
		Typhoid.	Para. A.	Para. B.
Between 1st and 4th day	...	11	12	8
" 5th " 8th "	...	51	46	12
" 9th " 12th "	...	26	20	2
" 13th " 16th "	...	8	3	3
17th day and afterwards	...	6	3	1

Assuming that the great majority of these cases arrived in hospital towards the end of the first week of the disease the distribution in time of the various diagnoses made would seem to show no material difference whether *B. typhosus* or *B. paratyphosus A* was the infecting organism. The figures for *B. paratyphosus B* are too small to be of significance.

Discussion and Conclusion.

Though I am unable to find any support for the assumption that in the inoculated individual who contracts enterica the degree and duration of the

bacteriæmia are altered to the consequent detriment of successful blood culture, I must admit the plausibility of the assumption. R. L. Stone⁵ has shown that typhoid bacilli injected intravenously into immunised rabbits disappear from the blood and tissues (except possibly the gall-bladder) in 24 hours, whereas in normal rabbits similarly injected bacilli may take ten days or more to disappear. Doubtless in the great majority of inoculated individuals who ingest typhoid bacilli some mechanism of this kind is at work to prevent infection. What part is played in this disappearance by bactericidal bodies in the blood and tissues must be left at present an open question in view of some recent divergence of opinion on the question of increased bactericidal antibody-content in the blood of animals immunised against *B. typhosus*. On the other hand, we must bear in mind that neither in the individual nor in the mass is immunity absolute. Even under the most rigidly controlled laboratory conditions, when large series of animals are taken, no antigen will produce an immunity which protects 100 per cent. of the immunised against a dose which kills 100 per cent. of the controls. The experiments of S. Rowland⁶ on immunisation of rats against *B. pestis* may be consulted on this point.

Under actual military conditions, although some approach to constancy of antigen and degree of immunisation may be realised, there can certainly be no constancy of the test dose or of the individual human factor. The results in the rabbit, an animal naturally insusceptible to typhoid fever and artificially educated to deal with *B. typhosus* introduced into its circulation directly, need not, in my view, have their strict counterpart in the vaccinated man who manages to contract by another channel of infection the germs of a disease to which his race is naturally susceptible. In the light of Besredka's recently promulgated views on immunity in enteric infections, the explanation may be found in inefficient immunisation of the intestinal epithelium or in a breakdown of what amount of immunity it may have acquired. The disease would then in all probability proceed on ordinary lines.

Vaughan (loc. cit.) in the search for possible explanations of the occurrence of typhoid in inoculated American troops, makes the following, among other suggestions. The vaccinated person takes into his interior probably large doses of the infecting bacilli, the majority of which he is capable of dealing with. Some, however, may find a nidus in the gall-bladder (as in the immunised rabbit) and there proliferate.

"The numbers of organisms that are continually discharged in the bile and resorbed through the intestinal mucosa call on the immunity mechanism for constant and exhausting action."

When to this antibody-exhaustion is added the fatigues and hardships of war with its attendant diarrhoeal troubles, the chances of an ultimate systemic infection are enhanced. This view, which would attribute a systemic infection to a breakdown of immunity in a carrier presupposes some evidence of intestinal trouble preceding the attack, and in this connexion Vaughan notes that of 104 inoculated men who contracted typhoid fever 60 per cent. had antecedent diarrhoea. It was Vaughan's opinion that two of the chief causes of typhoid fever among vaccinated American troops were (1) the ingestion of overwhelming doses of the infecting organism, and (2) loss of resistance and antibody-exhaustion due to the exposures of military life. It should be mentioned that the majority of Vaughan's cases had received the saline triple vaccine and a minority the lipovaccine. In a non-vaccinated population of all ages the variety of clinical forms in which enteric disease presents itself has long been recognised. Whether in a vaccinated population there occurs a relatively greater proportion of mild cases among the attacked than would be found among the attacked of a non-vaccinated population must be determined on a basis of cultural diagnosis. The practicability of such diagnosis is not, so far as present evidence shows, enfeebled by previous inoculation.

In conclusion I may add that I have been in communication with Sir William Leishman on the question ventilated in this note. Unfortunately it may be some time yet before the relevant data can be extracted from the medical case-sheets and laboratory files of the Western front. Sir W. Leishman informs me, however, that bacteriologists in France certainly supported the views I have attributed to them in the present communication. The question is of high theoretical and practical importance, and the publication of this note may, I hope, stimulate further inquiry.

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The Production of Coliform Infection in the Urinary Tract of Rabbits

BY

ELIZABETH H. LEPPER, M.B., B.S. (LOND.)

BEIT MEMORIAL RESEARCH FELLOW

From the Bacteriological Department of the Lister Institute, London

THE PRODUCTION OF COLIFORM INFECTION IN THE URINARY TRACT OF RABBITS.*

By ELIZABETH H. LEPPER, M.B., B.S. (Lond.), Beit Memorial
Research Fellow.

From the Bacteriological Department of the Lister Institute, London.

INTRODUCTORY.

THE earliest experimental work on the production of lesions in the urinary tract by means of coliform bacilli was done in the year 1888 by Albarran and Hallé (¹).

They found that cystitis occurred if injection of an emulsion of bacilli into the bladder was followed by ligature of the urethra. A similar result was obtained without obstruction of the urinary flow, if the mucous membrane of the bladder was injured by the introduction of a foreign body at the same time that the emulsion was injected.

They also produced pyelonephritis of one kidney by injecting organisms into the ureter, which was then ligatured; in two of these cases lesions were found in the opposite kidney as well.

These results were confirmed by Achard and Regnault (1891²), and by Schmidt and Aschoff (1893³).

Schnitzler (1890⁴) produced a cystitis by simply introducing the organisms into the bladder. The bacillus he used was a liquefier of gelatin, and intravenous injections of this organism were followed by intense nephritis. Schnitzler's results were at this time unique, no other observers having succeeded in producing cystitis without the infliction of some injury on the bladder mucous membrane in addition to the intra-vesical injection of organisms, and lesions in the kidneys following intravenous injections were only rarely described.

Bazy (1893⁵) ligatured the urethra and injected bacilli intravenously when a well-marked cystitis resulted. His experiments opened up a new field of research for subsequent investigators.

From 1886, following Wyssokowitsch's experiments, up to 1910, much work was done in order to determine whether organisms injected intravenously were excreted in the urine.

Wyssokowitsch (1886⁶) always found evidence of hæmorrhage or infarct in the kidney in those cases which had yielded positive urinary cultures. Boccardie and Pernice and Scagliosi (quoted by Jahn (1910⁷)), among others, confirmed these results.

Sherrington (1893⁸) concluded from his experiments that only organisms which are pathogenic for the animal used have the power of damaging the

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kidney and appearing in the urine—blood, hæmoglobin, or albumin being practically always present when positive cultures were obtained.

Vincenzi (1909⁹) got similar results except with a coliform organism which was excreted in the urine without any damage being detectable in the kidney.

None of these observers appear to have found any definite inflammatory changes in the kidneys resulting from their experiments—hæmorrhages or infarcts being the only lesions noted.

Wyssokowitsch, Sherrington, and Vincenzi collected the urine for examination from the bladder after the animal had been killed, various methods being adopted to avoid contamination.

Biedl and Kraus (1896¹⁰) investigated this subject again. They adopted a different technique, the urine being collected from the ureters by means of catheters specially designed so as not to injure the mucous membrane. They obtained results entirely contrary to those of Wyssokowitsch, bacilli appearing in the urine soon after the intravenous injection had been given. They found no evidence of renal hæmorrhage either by microscopic examination of the urine or in sections of the kidneys.

Their results were confirmed by von Klecki (1897¹¹) and Opitz (1898¹²) as well as by others. Opitz, however, found microscopic evidence of hæmorrhages in the kidneys.

Jahn attempted to explain these contradictory results by the difference in method of collecting the specimens employed by the two groups of observers. He found that the urine had a bactericidal action on a number of bacteria, this action being almost entirely due to the degree of acidity or alkalinity of the specimen. He attributed the negative results by Wyssokowitsch's method to the length of time that the organisms had been in contact with the urine in the bladder.

The possibility that the positive cultures obtained by the second group of observers may have been due to their method of collecting the urinary specimens by catheterisation of the ureters will be discussed later on in this paper.

Since 1911 the experimental production of coliform infections of the kidney has been the subject of considerable research in America.

Brewer (1911¹³) found that by damaging the kidneys he could render them susceptible to infection by way of the blood stream. The methods adopted were, ligature of one ureter, injection of a thick paste of bismuth subnitrate into the pelvis of one kidney, bruising the kidney by a blow on the back, or production of temporary anæmia by compression of the renal artery.

Hess (1913¹⁴) reinvestigated all the experimental methods which had been used previously and carried out much original work. He brought about a transient cystitis by simple injection of bacilli into the bladder. Much more severe lesions resulted if a preliminary injection of turpentine and paraffin were made, and these severe cases were associated with bilateral pyelitis. If a moderate degree of cystitis were set up and at the same time a partial narrowing of one ureter carried out, then a pyelitis was produced in the kidney from which the flow of urine was obstructed. No blood cultures are recorded in these cases. He also found that intravenous injection of bacilli led to pyelonephritis in a kidney if its ureter had been partially obstructed. This occurred whether the two operations were done at the same time, if the obstruction preceded the injection, or if the obstruction, having been in existence for six to eight days, was undone and an intravenous injection then made.

Helmholtz and Beeler (1917¹⁵) studied the effects of simple intravenous injection of coliform organisms. They produced lesions in the kidneys in eleven of the sixty-six rabbits used. In 1918, working with a bacillus isolated from a case of spontaneous pyelitis in a rabbit, they produced pyelitis in seventeen out of thirty-one rabbits. Thirteen of these also showed abscesses in the kidneys.

When this organism was given intravesically pyelitis resulted in ten of the fifteen rabbits used, while abscess in the kidney was found once. They state that the course of the infection could be traced along the lymphatics of the ureter to the pelvis of the kidney. Blood cultures at the autopsy were negative, but none are recorded as having been made during the course of the experiments.

David (1918¹⁶) made a number of experiments from which he concludes that "it is possible in an unobstructed bladder to infect the upper urinary tract by direct extension of the infection from the bladder through the lumen of the ureter."

The publications referred to indicate the methods used by the various observers who have been successful in producing lesions in the urinary tract. A full account of the literature can be found in Schmidt and Aschoff's monograph and in the papers of Hess and David.

From a consideration of the results that have been obtained, it appears that lesions in the urinary tract have been produced with ease by all observers who have combined intravenous injection of organisms with obstruction to the urinary flow; if this obstruction be in the ureter the kidney is infected, if in the urethra a cystitis is set up.

Pyelitis and pyelonephritis have been produced by intravenous injection of organisms without obstruction in the urinary tract, but the percentage of positive cases is not high with most of the bacilli used. There is some evidence that certain strains of *B. coli* have a selective action on the kidney.

There is no consensus of opinion as to the production of lesions in the kidney secondary to a bladder infection. Many attempts to effect this have been negative. In the experiments which have given positive results, there has been no systematic examination of blood cultures during the experiments, so that a generalised blood infection secondary to the cystitis has not been definitely excluded.

The following experiments were undertaken to produce lesions in the kidney by intravenous injection of coliform organisms in order to examine the inflammatory changes produced soon after infection, so that the path of the bacilli through the kidney might be studied, and further to devise some method whereby a kidney infection could be brought about with a reasonable degree of certainty.

THE LESIONS PRODUCED IN THE KIDNEYS OF RABBITS BY INTRAVENOUS INJECTIONS OF COLIFORM BACILLI.

Preliminary Experiments.

A number of experiments were carried out to determine the dose of coliform organisms sufficient to produce a blood infection but not sufficient to kill the rabbit. The organisms used had been isolated from cases of urinary infection in man. Representatives of MacConkey's first three groups were employed. The bacilli gave the following reactions; they all fermented lactose and gave the methyl-red reaction

of Clark and Lubs; they produced indol, and did not give the Voges Proskauer reaction or liquefy gelatin. The bacillus belonging to Group I. (prototype, *B. acidilactici*) did not ferment dulcitol or saccharose; grown on glycerine and salicin it produced acid and gas, and it was feebly motile.

The bacillus belonging to Group II. (prototype, *B. coli communis*) fermented dulcitol but not saccharose; on glycerine and salicin it produced acid and gas, and it was motile.

The bacillus belonging to Group III. (prototype, *B. coli communior*) fermented dulcitol, saccharose, and glycerine, but not salicin, and it was actively motile.

Emulsions of these organisms were made in sterile saline from 24-hour-old agar slopes and standardised to 2000 million bacilli per c.c. by Brown and Kirwan's (1914¹⁷) barium sulphate method. The injections were made into an ear vein. If the first injection failed to produce an infection of the kidney, increasing amounts of the bacillary emulsion were given at intervals of four to six days. It was recognised that in this type of experiment the resistance of the animal to infection might be increased by the repeated injections. The initial dose for the first three rabbits was 23 million organisms (*Series I.*), for the next three 200 million (*Series II.*).

The rabbits had a very definite rise in temperature after the injections, 104° F. to 106° F. being noted, representing a rise of one to three degrees above the normal temperature. There was no evidence, however, that the organisms were excreted by the kidney, and the twenty-four urine cultures all proved negative. The urine was collected by means of a glass catheter and cultures made three hours after an injection and again at the end of twenty-four hours.

Subsequently in two of the rabbits a lesion in the kidney was produced.

Production of Agglutinins.—In the course of these early experiments sera which agglutinated the stock cultures were obtained.

It was found that the serum of the rabbits which received the bacillus belonging to Group III. possessed a high agglutinating titre, and at the end of fourteen days the serum would give complete agglutination in a dilution of 1/1000. The bacilli of Groups I. and II. gave rise to agglutinins much more slowly, five to six weeks being necessary to obtain a serum which gave complete agglutination in a dilution of 1/1000. Similar results were obtained with other strains of these groups, but the reaction was not constant, some members of Group III. giving rise to only a small amount of agglutinin.

When the sera showed complete agglutination in a dilution 1/1000 for the homologous bacilli, they were tested against members of the other two groups in a dilution 1/100. Occasionally a slight agglutination was noted, but nothing in the least comparable to the result obtained with the homologous culture.

When tested against members of its own group some sera of Group II. agglutinated three out of eleven strains slightly, and some members of Group III. gave similar results with two out of four members of Group III. Even with

strains belonging to the same group there was only one instance of a serum giving a reaction at all comparable to that obtained with the homologous bacillus.

These agglutinating sera, therefore, supplied a fairly specific test for the organism in question, and always supplemented the sugar reactions in the identification of cultures recovered from the experimentally infected animals.

Experiments to test the Effect of Number of Organisms injected.

As the urine cultures from the first six rabbits proved negative, a larger initial dose was tried.

Series III.—In this third series of five rabbits 1000 million organisms were given. The results except for one positive culture on the fourth day were negative, and although one of these rabbits developed a severe blood infection, blood cultures being positive on the second and thirteenth days, urine cultures from this rabbit made on the first, fifth, ninth, and fifteenth days were all negative.

Series IV.—Four rabbits were then injected with an initial dose of 4000 million. One died within three hours, and one was killed at the end of twenty-four hours as it seemed very ill and likely to die. At the post-mortem the bacillus injected was recovered from 0.01 c.c. of the rabbit's blood. Urine cultures from both these rabbits were negative.

The remaining two rabbits seemed ill for several days. One gave a negative blood culture using 0.1 c.c. at the end of three and twenty-four hours, but was positive in 0.01 c.c. on the second day. The method adopted was to collect blood from an ear vein with a sterile all-glass syringe, transfer it to a sterile test-tube, and then to draw up 0.1 c.c. into a sterile 1 c.c. pipette graduated in hundredths, and mix it immediately with 0.9 c.c. of citrated broth which had been measured out ready in another tube. 0.1 c.c. of this 1 in 10 dilution was then carried over into another tube containing 0.9 c.c. of citrated broth, and so on till a dilution of 1 in 10,000 had been made.

The other rabbit gave a positive blood culture in 0.005 c.c. at the end of twenty-four hours, and in 0.1 c.c. on the second day.

Both rabbits gave negative blood cultures on the fourth day when 0.25 c.c. of blood was incubated in 5 c.c. of citrated broth.

Urine cultures made from these rabbits one hour, twenty-four hours, two, four, seven, nine, and thirteen days after the injection were all negative.

The results of these experiments show that bacilli are not as a rule excreted by the kidneys of an animal suffering from a coliform blood infection, even though the organisms may be present in the circulation in considerable numbers.

In the course of these experiments with fifteen rabbits three animals developed lesions in the kidney, two of which proved to be due to coliform infection. In two the lesions were found post-mortem, while the third rabbit was watched throughout the course of its disease. The particulars of this third rabbit are as follows:—

The rabbit was given 240, 600, and 920 million organisms intravenously in the course of ten days. During the last injection some of the emulsion was accidentally injected round the vein with the result that the rabbit developed a small ulcer on its ear; its temperature for three days remained between 104° F.

and 105° F. On the third day a blood culture was positive. Urine collected on the following day contained pus cells and cellular casts but no bacteria were seen. The culture proved positive. After the sixth day the temperature returned to its normal level. At the end of a fortnight the urine still contained pus cells and hyaline casts, and culture was positive.

A further injection of 1200 million organisms was given. After this the urine was examined once or twice a week for five weeks. The cultures were positive throughout this period; pus cells, hyaline, and granular casts were present, and bacilli were also found in large numbers a fortnight after the infection was first noted. In the seventh week the cultures became negative though a few leucocytes and epithelial cells were found up to the eleventh week.

The rabbit remained in good health during the course of the disease and gained in weight. It was killed two months after the cultures became negative. Sections of the kidneys and bladder were made. Those from the right kidney showed a slight increase in the connective tissue of the papilla and small collections of degenerate polymorphonuclear leucocytes were found underlying the mucous membrane of the parietal wall of the pelvis. The sections of the left kidney and bladder showed no change.

The other two rabbits had given negative urine cultures after the early injection, and appeared in good health; there was no rise of temperature to suggest that an infection had occurred.

The lesions found post-mortem were in one a right-sided pyelitis from which the organism injected (Group II.) was isolated in pure culture, and in the other rabbit a wedge-shaped abscess of the cortex with its apex pointing towards the medulla. From this abscess *Staphylococcus aureus* was grown and sections stained for organisms showed Gram-positive cocci only, so that this lesion must have been due to an intercurrent staphylococcal infection.

The Importance of the Strain of Bacillus used.

In connection with these experiments twelve different strains of *B. coli* were used, ten derived from cases of urinary infection and the remainder from faeces.

One organism, "Styles," belonging to Group III. produced, after an initial dose of 1000 million, an infection similar to that already described. The particulars of this rabbit are as follows: The urine twenty-four hours after the injection contained some pus and epithelial cells and a few bacilli. On the following day there was an enormous number of bacilli in the direct films, comparatively few pus cells, a large amount of epithelium, and some hyaline casts. Cultures from the urine continued to be positive till the end of a week, when the condition cleared up entirely. The rabbit lost 120 grms. in weight during the week, but rapidly improved in condition when the cultures became negative.

The ease with which infection was obtained with this organism was so impressive that an attempt was made to determine whether it had a selective action on the kidney.

Eight young rabbits were available, varying in weight from 650 to 950 grms. Doses from 600 to 1000 million in proportion to weight were injected. The dose employed proved to be excessive for these small animals. Two died within three hours, one in twenty-four hours, and the remainder were killed on the fourth day. Three of these gave positive urinary cultures, and microscopically four of the five showed kidney lesions.

The unusually large number of positive results in this experiment may have been due to a selective action of the organism used, but it is possible that the age of the rabbits was an etiological factor. So far the action of this organism has not been further studied.

The Localisation of the Kidney Lesions.

The microscopical appearances found in the kidneys of these rabbits threw light on the urinary changes noted in the previous rabbits. The inflammatory changes were almost entirely confined to the papilla of the kidney. In the most marked cases there was necrosis of the tip, and of the epithelium covering it, the cells of the tubules being swollen, staining badly, and the body of the cell being entirely filled with organisms. There were a few leucocytes between these degenerate cells and a fair number in the interstitial tissue between the tubules. The sections made from the rabbit which died twenty-four hours after injection showed that some of the capillary blood-vessels supplying the papilla were packed with bacilli. These vessels were surrounded by polymorphonuclear leucocytes.

The sequence of events in coliform infections of the kidney following intravenous injections so far as they could be traced in these sections is, therefore, first a bacillary embolism of capillary vessels, generally those of the papilla, followed by a round cell infiltration in the connective tissue surrounding the affected vessels; if the infection is severe the inflammation spreads to the tubules, the epithelial cells take up the bacilli in large numbers, many of those cells die and desquamate, pus, epithelium and bacteria collect in the tubules, and in some cases necrosis of the tip of the papilla may occur. The inflammation in some cases subsides and even microscopically may leave very little trace.

THE PRODUCTION OF LESIONS IN THE KIDNEYS OF RABBITS BY INTRAVENOUS INJECTION OF COLIFORM BACILLI COMBINED WITH OBSTRUCTION OF THE URETER.

An attempt was made to see if any difference could be detected in the kidneys which had been infected by way of the ureter as compared with those in which the bacteria had reached the kidney through the blood stream.

In the cases now to be described the ureter of the left kidney was

exposed by an incision in the loin and an emulsion of bacilli injected into its lumen, a ligature being then tied above the puncture wound; or an injection of organisms having been given into an ear vein, the ureter was exposed and tied. The animals were killed six days later. In all cases the kidneys showed very marked diffuse inflammatory changes, but as all the rabbits which had received intra-urethral injections gave positive blood cultures, it was not considered practicable to attempt to differentiate lesions produced in this way.

The inflammatory lesions found in the kidney when intravenous injections had been combined with ligature of the ureter were so marked that it seemed worth while to attempt to find out how long the obstruction to the urinary flow must be maintained in order that an infection of the kidney might be brought about.

The method adopted was as follows. The left ureter was exposed by an incision in the loin and freed sufficiently to be drawn up into the wound. An elastic band, a third of an inch wide, was passed under it, and the band drawn tightly over the ureter by fastening it with a pair of bulldog forceps. The lumen of the ureter was completely obstructed. The compression was applied about $\frac{3}{4}$ inch below the pelvis of the kidney.

Details of Experiments:—*Exp. I.*—Six hundred million bacilli were injected into the ear vein of a rabbit and three hours later the ureter was exposed and compressed for fifty-five minutes. The ureter was then carefully replaced and the muscle and skin sutured.

The rabbit seemed well for the first two days, but on the third was cold and had diarrhoea. It was killed on the fourth day. At the post-mortem the left kidney was not enlarged; on the anterior surface there were a large number of white specks of the size of a pin's head and slightly raised. In places these had run together, giving rise to white patches. The posterior surface showed similar but less marked changes. On section there were numerous white streaks running from the cortex to the medulla. Portions of the medulla looked necrotic, and were surrounded by a zone of congestion. The pelvis was not dilated but the surface of the papilla was covered with pus.

The right kidney appeared healthy, but on section pus was seen in the pelvis. The spleen was enlarged and contained a number of infarcts; the liver also contained a few infarcts, and some were present at the base of the left lung.

Cultures from the blood, bladder, both kidneys, and spleen were positive.

Exp. II.—The ureter was exposed and all bleeding points secured; 1000 million organisms were then injected into an ear vein and the ureter compressed for thirty minutes.

The animal was killed on the fourth day.

At the post-mortem the left kidney was found to be three times its normal size, the surface was covered with closely packed white granules the size of a pin's head. The capsule stripped easily. Section of the kidney showed many white streaks running down to the medulla. There were also wedge-shaped areas of necrotic-looking tissue having their apices directed towards the medulla. The pelvis was not dilated. The connective tissue surrounding the upper part of the ureter was swollen and cedematous. The ureter was adherent to the lower pole of the enlarged kidney and slightly bent, but did not appear to be obstructed, being wider than that of the right side and full of pus. The right kidney showed nothing abnormal.

Microscopic sections of the bladder showed inflammation of its mucous membrane.

Exp. III.—A dose of 500 million organisms was given and the ureter compressed for fifteen minutes. The animal was killed on the fifth day and showed nothing abnormal.

Exp. IV.—The rabbit had a dose of 1000 million and the ureter was obstructed for fifteen minutes.

At the post-mortem on the seventh day the left kidney was of normal size. On the anterior surface was a raised nodule 3 mm. in diameter. On section this area of the cortex was pale and white streaks ran down to the medulla. The pelvis was not dilated.

The urine culture was positive. The right kidney showed nothing abnormal.

Exp. V.—A dose of 600 million was given and the ureter obstructed for thirty minutes. A urinary culture made immediately after the operation was negative. On the second day the urine contained pus cells and casts, and the culture was positive. On the sixth day pus cells and very numerous bacilli were present. The urine taken on the ninth, thirteenth, and twentieth days showed the same appearances. The animal was then killed.

At the post-mortem both kidneys looked quite healthy. Sections of the right kidney and bladder were normal, the left kidney showed a slight round cell infiltration of the mucous membrane covering the papilla.

In these five experiments the bacillus belonging to Group I. was used.

Exp. VI.—In this rabbit the ureter was exposed and 600 million of the bacillus belonging to Group III. injected intravenously. The ureter was compressed for fifty-five minutes.

The animal was killed on the sixth day. At the post-mortem the appearance of the left kidney was almost identical with that described in *Exp. II.*

The right kidney served as a control in these experiments, for presumably it was of the same susceptibility and had been subjected to the same dose of organisms and the same amount of anæsthetic.

THE MECHANISM BY WHICH INCREASED PRESSURE IN THE PELVIS DAMAGES THE KIDNEY.

The appearances found in a rabbit which died of shock during the course of an experiment suggest that the lesions are secondary to hæmorrhages following over-distension of the veins owing to the obstruction to the venous return caused by the pressure of the distended pelvis on these thin-walled vessels.

The details of this experiment are as follows:—

The ureter was exposed and 600 million organisms injected, intravenously, and the ureter was compressed. Fifteen minutes later the rabbit died of shock which developed very suddenly, the pulse being good until the breathing became laboured about two minutes before death. The ureter was tied above the site of compression, the renal vessels ligatured, and the kidney removed and placed in 4 per cent. formalin. On cutting into the kidney after fixation the pelvis was found to be dilated, and contained some blood clot. The lateral walls of the pelvis were pressed out between the renal vessels so that these appeared as strands running across the pelvis. They must have been subjected to considerable lateral pressure. Microscopic sections showed that the veins of the kidney were greatly distended.

In a rabbit which was killed by ether for the purpose of studying the changes in the kidney at the end of one of these experiments, no dilatation of the pelvis was present although in this case the ureter had been compressed for thirty minutes. It is probable, therefore, that the occurrence of infection depends on the excretory activity of the kidney during the period that the ureter is compressed. If no urine is excreted, no dilatation of the pelvis occurs, and no obstruction of the veins results.

The observations of Lucas (1908¹⁸) and of Draper (1913¹⁹) on the effects of increased pressure in the pelvis of the kidney are of interest in this connection. Lucas, working on the physiology of the ureter, found that a rise of pressure in the pelvis was associated with a diminished flow of blood through the kidney, and Draper states that hæmaturia is always obtained when the pressure in the renal pelvis is raised.

How long the effect of obstruction to the urinary flow lasts has not been determined. Only one experiment has been made.

The left ureter was compressed for an hour. The rabbit was allowed to recover from the anæsthetic and three hours later given 1000 million organisms intravenously. One positive culture only was obtained from the urine on the third day after the operation. A subsequent dose of 2000 million was without effect, and when the rabbit was killed two and a half months after the operation the kidney was apparently quite normal.

The marked influence which temporary obstruction to the flow of urine appears to have on the kidney may be the explanation of the divergent results obtained by different observers on the question of passage of organisms from the blood into the urine.

The manipulation necessary to secure the ureteral catheters which were used by Biedl and Kraus, and also by Opitz, who obtained positive cultures from the urine after intravenous injection of various organisms, may have led temporarily to an increased pressure in the pelvis of the kidney, and slight hæmaturia may have resulted (1 c.c. of urine containing 0.001 c.c. of blood looks quite clear, but that amount of blood has in some cases been found to be sufficient to give a positive blood culture, even after the comparatively small doses of organism given in my experiments).

Biedl and Kraus also noted that sugar solution and normal saline, of which they gave from 100 to 400 c.c. intravenously during the course of their experiments, increased the positive findings. If some degree of obstruction was present in the ureters of their experimental animals, the increased urinary flow produced by these injections would materially assist in bringing about a dilatation of the pelvis of the kidney.

The positive results they obtained by continuous drainage of the bladder cannot be explained in this way.

These experiments show that the production of a temporary hydronephrosis combined with the presence of coliform organisms in the blood stream can lead to a generalised infection of the kidney which is similar in type to that found at post-mortem examinations on fatal cases occurring in man.

The mode of production of these lesions is probably hæmorrhage into the kidney from dilated veins caused by the pressure of the distended pelvis on the renal vessels.

EXPERIMENTS TO INFECT THE KIDNEY DIRECTLY FROM THE SURROUNDING CONNECTIVE TISSUE.

A small collection of pus was sometimes found round the stitches which had been put in the layer of muscle in the rabbits operated upon. It was thought possible that the organisms in these cases might have spread along the lymphatics from the wound to the kidney, and that this was the explanation why the left kidney was affected.

Exp. I.—Two young rabbits were injected intramuscularly in the left loin as nearly as possible at the usual site of the muscular incision with 400 and 800 million organisms respectively.

One died during the second night, and the other was killed on the fourth day. Cultures from the latter were positive from muscle, but negative from blood and urine.

Both rabbits showed extensive infiltration of the retroperitoneal tissues, with slight exudation of lymph on the peritoneal surface. The infiltration spread up round the lower pole of the left kidney.

The kidneys appeared healthy in both cases. Microscopically the cellular tissue round the lower pole of the left kidney was crowded with organisms. Just outside the kidney capsule there was a thick layer of leucocytes in which bacteria were difficult to find. The vessels of the capsule and surface of the cortex were dilated and congested, but no organisms could be found and no round cell infiltration could be detected in cortex or pelvis. Cloudy swelling of the convoluted tubules was present.

In these two rabbits, although a very large number of organisms were in close proximity to the kidney, no inflammation of that organ had occurred.

Exp. II.—Two larger rabbits weighing 1890 and 1620 grms. were given 850 and 680 million organisms respectively intramuscularly in the left loin, care being taken not to penetrate quite so deeply as in the first two rabbits.

Blood cultures and urine cultures were made daily, 1 c.c. of blood being inoculated into 5 c.c. of citrated broth.

Blood cultures were all sterile in the one rabbit.

Urine cultures were positive on the sixth and eighth days when a cloud of albumin was present, but no pus cells were found.

The rabbit seemed out of sorts on the third to fifth day. It was killed on the tenth day. At the post-mortem there was considerable necrosis of the erector spinæ muscle, the kidneys looked normal, and no microscopical changes could be detected.

The second rabbit gave a positive blood culture on the fourth day. Albumin,

pus cells, and casts appeared in the urine on the sixth day. Bacilli were found in large numbers on the eighth day. The cultures continued positive and the animal was killed on the sixteenth day after the onset of the urinary infection.

At the post-mortem the kidneys appeared normal. Microscopically there were well-marked inflammatory changes in the papilla of each kidney, but no cortical abscesses were present.

In neither of these rabbits was there any evidence of a direct or lymphatic spread to the kidneys; there was, however, in one of the rabbits, a blood infection which preceded the positive urinary culture by two days.

CONCLUSIONS.

1. When coliform bacilli appear in the urine after intravenous injection of these organisms, there has always been, with only one exception, evidence of accompanying kidney changes as shown by the presence of albumin, or pus, epithelium, and bacilli in the urine.

2. The complete obstruction of the urinary flow from a kidney for so short a period as fifteen minutes is sufficient to make it vulnerable to coliform organisms circulating in the blood.

3. There is some evidence that the type of inflammation resulting from blood infection combined with obstruction to the urinary flow is more severe than that produced by blood infection alone.

4. Severe infections of the connective tissue in the region of the kidney do not spread very rapidly to that organ, but if a blood infection results from the septic focus a kidney lesion may follow.

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HÆMAGGLUTINATION AND ITS MEDICO-LEGAL BEARING, WITH OBSERVATIONS UPON THE THEORY OF ISOAGGLUTININS.

H. SCHÜTZE, M.D.

From the Bacteriological Department, Lister Institute, London.

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THE precipitin test has long been accepted as a means of differentiating one species of blood from another. Should the specimen be a human one, it might, however, be of the greatest medico-legal importance to carry the investigation one step further, and ascertain the iso-agglutination group* to which the specimen belongs. The necessity of investigating the possibility of grouping according to Moss (¹) blood which is already dry arose for the author in connection with a case where material, alleged to be blood supernaturally pouring from a holy picture, was found to react positively with anti-human precipitin serum, and it was desirable to exclude or include certain persons as possible sources of supply.

No work appears to have been done to establish the possibility of grouping blood that has dried, or to find out for how long after desiccation such grouping might be practicable, but that a serum reconstructed from freshly dried blood still contains hæmagglutinins is seen from the following experiment:

Experiment I.—One c.c. of a Group II blood dried overnight in a Petri dish was reconstructed the following morning by rubbing up the material in 1 c.c. of distilled water with pestle and mortar and centrifuging to get rid of the undissolved residue. The hæmoglobin-stained supernatant was then used, alongside of serum obtained from the same individual, for the agglutination of red cells of known type.

Serum.	Red cell.	Serum dilutions.							
		$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	
Group II (Fresh)	Group I	†	†	†	†	†	+	—	
	Group III	†	†	†	†	†	+	—	
Group II (Reconstructed after 1 day)	Group I	†	†	†	†	+	—	—	
	Group III	†	†	†	†	+	—	—	

(† = macroscopic clumping; + = microscopic clumping.)

* The reactions of the Moss groups are as follows:

Serum.	I.	II.	III.	IV.
Cell I	—	+	+	+
Cell II	—	—	+	+
Cell III	—	+	—	+
Cell IV	—	—	—	—

(+ = agglutination of red cell by serum.)

reconstructed blood, the centrifuged deposit left over from Experiment I was washed in saline, and re-suspended in 0.5 c.c. of the same fluid. Of this suspension 0.3 c.c. was added to 0.3 c.c. of Group IV (D)* serum, and the mixture allowed to stand for 2 hours at room temperature, being shaken from time to time. As the Group II cells (R) were known from previous experience to be less agglutinable than certain others also belonging to Group II, absorption of a second quantity of Group IV (D) serum was similarly carried out with the undissolved residue from the more agglutinable Group II cells (F), and agglutination demonstrated with both homologous and heterologous examples of well and less well agglutinating Group II cells. Corresponding absorptions, but with fresh washed cells (as in Experiment II) from the same persons, were undertaken at the same time.

Serum IV (D).	Unabsorbed.						Absorbed with Cell II (F).					
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$
Cell I (J)	+	+	+	+	+	—	+	+	+	+	—	—
Cell II (S)	+	+	+	+	+	—	+	—	—	—	—	—
Cell II (F)	+	+	+	+	+	—	+	—	—	—	—	—
Cell II (A)	+	+	+	+	+	—	—	—	—	—	—	—
Cell II (R)	+	+	+	—	—	—	—	—	—	—	—	—
Cell III (Z)	+	+	+	—	—	—	+	+	+	—	—	—

Serum IV (D).	Absorbed with Residue II (F).						Absorbed with Cell II (R).					
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$
Cell I (J)	+	+	+	+	—	—	+	+	+	+	+	—
Cell II (S)	+	—	—	—	—	—	+	+	+	+	+	—
Cell II (F)	+	—	—	—	—	—	+	+	+	+	+	—
Cell II (A)	—	—	—	—	—	—	+	+	—	—	—	—
Cell II (R)	—	—	—	—	—	—	+	—	—	—	—	—
Cell III (Z)	+	+	+	+	—	—	+	+	+	—	—	—

Serum V (D).	Absorbed with Residue II (R).					
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$
Cell I (J)	+	+	+	+	+	+
Cell II (S)	+	+	+	+	+	—
Cell II (F)	+	+	+	+	+	+
Cell II (A)	+	+	+	—	—	—
Cell II (R)	+	—	—	—	—	—
Cell III (Z)	+	+	+	—	—	—

It will be seen that absorption is as successful with blood residues as it is with fresh cells, and badly agglutinating cells are, as might be expected, also badly absorptive ones; but although saturation with such badly agglutinating Group II cells does not alter the titre of the serum for the better agglutina-

* Bracketed initials indicate the individual from whom the material was obtained.

ting Group II cells (S) and (F), the titre as registered by the more or less poorly agglutinating Group II cells (A) and (R) is definitely affected.

The recognition of the existence of poorly agglutinating Group II cells—(the cells of 5 individuals out of 13 of Group II have been found to possess this characteristic in more or less marked degree; on the other hand, those of 9 persons of Group III which have been tested were all equally agglutinable)—is of importance for two reasons. When a serum whose titre may from one cause or another have suffered is being grouped, it is obviously advisable to choose a cell with maximal agglutinating powers; while if cell-suspension or residue of unknown nature is being grouped by the absorption method, it is, on the contrary, better to employ a weak cell for agglutination, as being a better general indicator of an eventual decrease in the agglutinin content of the serum.

The possibility of grouping dried blood by agglutinating with its reconstructed serum and absorbing with the cell residue has been demonstrated, but it still remains to be seen how long the antigens and antibodies concerned remain diagnosable; this must no doubt depend on the original titre and the influences to which the dried blood has been subjected.

The present observations extend over a number of months, and grouping has been possible in every case.

In Experiment IV a low-titred blood is examined after 41 days' exposure to sunlight, the specimen being dried on cloth.

In Experiments V and VI high- and low-titred sera are examined 5 months after drying; in each case serum taken at the same bleeding and preserved bottled, in the cold room, is examined for an indication of the keeping qualities of hæmagglutinin sera.

Experiment IV.—Blood of Group III (St) was taken; 0·5 c.c. was dried on a piece of clean linen and exposed to the air on a shelf near a window, where much indirect and some direct sunlight fell upon it, and reconstructed as usual by rubbing and squeezing in a mortar, *but with* 1 c.c. of distilled water, after a period of 41 days.

Agglutination and absorption tests resulted as follows:

Serum III (St).	Fresh.				Reconstructed after 41 days on cloth exposed to sunlight.		
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$
Cell II (S) . . .	†	†	†	—	†	†	—

Serum IV (M).	Unabsorbed.					Absorbed with Residue III (St), reconstructed after 41 days.				
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$
Cell I (J) . . .	†	†	†	+	—	†	†	—	—	—
Cell II (S) . . .	†	†	†	+	—	†	†	+	—	—
Cell III (Sa) . .	†	†	+	+	—	—	—	—	—	—

The titre has fallen slightly but the insoluble residue still shows marked avidity for its corresponding Group III cell agglutinins.

Experiment V.—In Experiment V blood of Group II (R) was taken; this individual constantly yields a high-titred serum with cells of poor agglutinability. One c.c. dried in a Petri dish was reconstructed and titred the following day; two similar specimens, one kept in a cupboard sheltered from light and the other exposed to it at a window, and both at room temperature, were reconstructed after 5 months, and examined together with a sample of the same bleeding kept, *as serum*, bottled and in the cold room.

The following titres were obtained :

Blood.	Particulars.	Titre against Cell I (J) and Cell III (Z).
II (R) undried . .	Serum at time of bleeding	$\frac{1}{64}$
„ dried . .	Reconstructed after 1 day	$\frac{1}{32}$
„ „ . .	Reconstructed after 5 months, sheltered from light	$\frac{1}{8}$
„ „ . .	Reconstructed after 5 months, exposed to light	$\frac{1}{4}$
„ undried . .	Serum after 5 months in the cold room	$\frac{1}{32}$

The five-months-old dried blood still retains sufficient agglutinin to make the grouping of it an easy matter; even the specimen freely exposed to sunlight, though of a lower titre than the one preserved in the dark, yields a serum capable of agglutinating Group I and III cells in a dilution of 1 in 4. The serum preserved for diagnosis purposes in the cold room has lost during the same period but little of its agglutinating power.

Experiment VI.—In Experiment VI, blood Group II (F) was used; the donor of this has, in contra-distinction to (R) of the previous experiment, always been found to have a low-titred serum with easily agglutinable red cells, but this inverse ratio of agglutinability of cell to titre of serum does not appear to hold good in all cases. One c.c. of Group II (F) was dried and reconstructed after one day; a further c.c. was dried, exposed to sunlight for 5 months and then examined; in both cases the absorption power of the insoluble residue was tested. The keeping qualities of a low-titred serum at 0° C. was also observed.

Blood.	Particulars.	Titre against Cell I (J) and Cell III (Z).
II (F) undried . .	Serum at time of bleeding	$\frac{1}{8}$
„ dried . .	Reconstructed after 1 day	$\frac{1}{4}$
„ „ . .	Reconstructed after 5 months, exposed to light	$\frac{1}{1}$
„ undried . .	Serum after 5 months in the cold room	$\frac{1}{4}$

Serum IV (D).	Unabsorbed.						Absorbed with Residue II (F), reconstructed after 1 day.					
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$
Cell II (S) . . .	+	+	+	+	+	—	+	—	—	—	—	—
Cell II (R) . . .	+	+	+	—	—	—	—	—	—	—	—	—
Cell III (Z) . . .	+	+	+	—	—	—	+	+	+	+	—	—

Serum IV (D).	Unabsorbed.						Absorbed with Residue II (F), reconstructed after 5 months, exposed to light.					
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$
Cell II (S) . . .	+	+	+	+	+	+	+	+	+	+	—	—
Cell II (R) . . .	+	+	+	+	—	—	—	—	—	—	—	—
Cell III (Z) . . .	+	+	+	+	—	—	+	+	+	+	—	—

The low-titred blood Group II (F) after 5 months' exposure to light in a desiccated condition has just sufficient agglutinating power left to indicate the group to which it belongs when tested in undiluted strength.

The complementary absorption test, however, establishes the group with more definiteness, although the specimen shows deterioration in this respect, too. Here again it is seen that less readily agglutinated cells, such as Group II (R), serve best as an indication of absorption. While the titre of the Group IV (D) serum is only somewhat lowered for the easily agglutinable cell Group II (S), that for cell Group II (R) is more markedly affected; the alien groups cells III (Z) agglutinate to the same extent in both absorbed and unabsorbed sera.

That portion of the Group II (F) bleeding kept, *as serum*, in the cold shows little depreciation in its agglutinating capacity.

It can be stated, then, that both antigen and agglutinin continue to be maintained for months in diagnosable form in dried blood specimens, and that probably in those blood-stains which have suffered most from adverse conditions, the absorption of a Group IV serum with the insoluble residue obtained when the dried blood is reconstructed will be the more successful diagnostic method.

With regard to the keeping properties of hæmagglutinating serum for diagnostic purposes, it may be mentioned here that the addition of 0.5 per cent. phenol, as is customary in the case of bacterial agglutinating sera, is not to be recommended. There is evidence that under the influence of the preservative the titre tends to sink.

Kolmer (⁶), working with serum, *not whole blood*, has found that samples dried on filter-paper deteriorate after 3 days in the cold room—how markedly one cannot tell, as the results given are qualitative only and not quantitative.

SUMMARY.

The possibility of grouping dried human blood specimens by reconstructing the serum for agglutination and using the undissolved residue for absorption has been demonstrated. Forensically the test would be of most importance when proving dissimilarity between two specimens alleged to be derived from the same source. To prove their similarity would probably only be to furnish circumstantial evidence of more or less value according to the group in question, and the frequency of the occurrence of that group in the population concerned.⁽⁷⁾

The Landsteiner theory that two substances, "A" and "B," with their corresponding agglutinins, "a" and "b," are concerned in the isoagglutination of human bloods has been confirmed by absorption tests.

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THE PRESENT POSITION OF VITAMINES IN CLINICAL MEDICINE.

*Being Contributions to a Discussion in the Section of Medicine
at the Annual Meeting of the British Medical Association,
Cambridge, July 1st, 1920.*

I.—HARRIETTE CHICK, D.Sc.,

LISTER INSTITUTE.

As a laboratory worker I should in any case feel very diffident about addressing an audience such as this, and feel even more humble for the reason that, during the last nine months in Vienna, I have seen something of practical medicine and have acquired a very wholesome respect for clinical work and investigation. I have discovered for myself that laboratory research is a comparatively simple and easy matter when compared with clinical investigation. And in our present endeavour to discover the part played by accessory factors in clinical medicine, I feel sure we shall need the best united efforts of both the clinician and the laboratory investigator, working together with mutual forbearance and unlimited goodwill.

I propose to leave to my colleague, Dr. Elsie Dalyell, any description of our clinical experiences in Vienna, but it would perhaps be helpful to the discussion if I stated the general principles of nutrition, gleaned from the results of laboratory work with animals, which we took with us as a guide when we set out to study hunger and deficiency diseases in Austria. We considered it to be a proved fact that for satisfactory nutrition of animals the diet must have an adequate calorie value and must contain a sufficient amount of the following constituents.

1. Mineral salts.
2. Carbohydrates.
3. Fats.
4. Protein, adequate in amount and of suitable nature.
5. Water-soluble or antineuritic (anti-beriberi) accessory factor or "vitamine."
6. Fat-soluble accessory factor.
7. Antiscorbutic accessory factor.

We believed that in the young growing mammal growth could be checked by a deficiency in any one of these seven constituents, although an ample supply of the

other six were present. We also had evidence showing that definite "deficiency" diseases could be developed in both young and adult after prolonged deprivation of any one of the constituents 4, 5, 6, or 7. Thus, after deprivation of No. 4 we might expect something like pellagra to develop; after deprivation of No. 5, tropical beri-beri or perhaps some other nervous disorder; after deprivation of No. 6, rickets, osteomalacia, hemeralopia, or keratomalacia in infants; after deprivation of No. 7, scurvy.

I must admit that all this is a diagrammatic representation of the facts, and that our clinical observations during the last few months have not grouped themselves with the exactness of the scheme as I have drawn it. But it is true to say that very often our cases did fall naturally into the places we had prepared for them.

There was another general conception we took with us from the laboratory, and this proved to be perhaps the most valuable guide we had. It was the importance of *quantity* when dealing with accessory factors in nutrition. This idea helped us to a solution of many difficult points encountered in our clinical observations. It is not enough, for example, to know that the "fat soluble" accessory factor is present in a diet; there must be enough of it. This factor can be presented in many different animal and vegetable fats, and to obtain an adequate amount of the vitamine the amounts of these fats would have to be different. Almost any fresh vegetable or fruit can be employed as antiscorbutic in a diet, but the minimal amounts necessary for protection from scurvy will be different. May I quote an example? Some children in our charge were getting 50 grams daily of raw apple juice as an antiscorbutic, and to our surprise began to show symptoms of a kind which we had learned to associate with the first onset of scurvy. We had no exact experimental data as to the antiscorbutic value of raw apple juice; we only knew that it was much inferior to the juice of lemons and many other fruits. We substituted 10 grams daily of raw neutralized lemon juice with excellent results.

Our observations in Vienna, then, confirm us in the belief that foodstuffs must not be labelled as containing, or not containing, these factors, but as containing them in different degrees. This is little understood. Even a physician will assure you that an infant gets "plenty of antiscorbutic" material in its diet of cow's milk, but will be unable to say for how long or to what temperature the milk is being heated. You will be told that a child is getting "plenty of milk fat" in its diet, but be unable to ascertain whether it is receiving 20, 30, or 40 grams daily. Our experience during the last few months has led us to believe that a difference between, say, 25 and 30 grams daily may have an important bearing upon the growth and development of an infant.

In Vienna we found large numbers of abnormal and diseased infants and young children, suffering from scurvy, rickets, and delayed growth and development. There

was no deterioration in general hygienic conditions to account for this fact, and we attribute it to malnutrition caused by the state of partial famine obtaining in Vienna. The same state of things prevailed in children at home and also in institutions, where no lack of calories could be detected in the diets provided, but a great deficiency in accessory factors. Shortage of animal and other fats was, perhaps, the most marked defect in the diet of the pregnant and nursing mothers. As regards artificially-fed infants, disorganized transport, necessitating the heating of milk once or even twice before delivery, reduced the antiscorbutic value of the fresh milk available, while partial substitution of milk by sugar and cereals caused a diminution of both "fat-soluble" and antiscorbutic accessories in the diet. Severe infantile scurvy was a common occurrence at all times of the year, and rickets was almost universal in infants of nine months and upwards. I will leave Dr. Dalyell to describe some of our attempts to prevent and cure these conditions by supplying accessory factors to the diets.

II.—ELSIE J. DALYELL, M.B. (BEIT MEMORIAL FELLOW), SYDNEY, AUSTRALIA.

In our recent work among children in Vienna the following were among the most important features from a medical point of view:

1. An unusual frequency of infantile scurvy, or "Barlow's disease" as it is called in Central Europe. This disorder occurred at all times of the year and in all stages of severity. There were cases showing the severe characteristic symptoms described in all textbooks, as well as many showing no definite symptoms at all beyond general ill health and failure to grow and develop. In these cases the only method of diagnosis lay in observing the effect of adding antiscorbutic material to the diet.

2. Great prevalence of rickets in both breast-fed and artificially-fed children. Marked stigmata of rickets were to be observed at a much earlier age than is usual. In one community, which included many breast-fed infants, rickets was diagnosed in 50 per cent. of the infants at 5 months and in 100 per cent. at 9 months. Accompanying this great increase in general incidence of rickets there was much dwarfism and severe deformity in older children.

3. Marked delay in growth and general development, either with or without the definite appearances of rickets or scurvy.

We confined our attention principally to children in certain institutions where, as the result of much devoted effort, the authorities had succeeded in providing a diet containing adequate calorie value. In these institutions, according to the admirable system still maintained in spite of present difficulties, accurate records are kept of medical history, of the diet, and of the general development of the little inmates. In many cases a study of these records suggested that deficiency of accessory factors in

the diet of the child (or of the mother in case of breast-fed infants) might be, at least in part, responsible for the condition. With the co-operation of the authorities we were permitted to try the effect of enriching the diet with accessory factors in cases where it appeared desirable, and we wish to record our thanks to Professor Clemens von Pirquet, University Kinderklinik; to Director Dr. Riether, Landes Zentral Kinderheim, and to Dozent Dr. Moll, Reichsanstalt für Säuglinge, for the facilities afforded us in the institutions under their respective control. The following charts (shown on the screen) illustrate six typical instances selected from a large number, showing the result of adding to the diet antiscorbutic and fat-soluble accessory factors alone or in combination.

Chart 1.—A weight chart showing marked improvement in growth and development of a breast-fed child after addition of 30 grams raw turnip juice and 50 grams of butter daily to the mother's diet. When the treatment

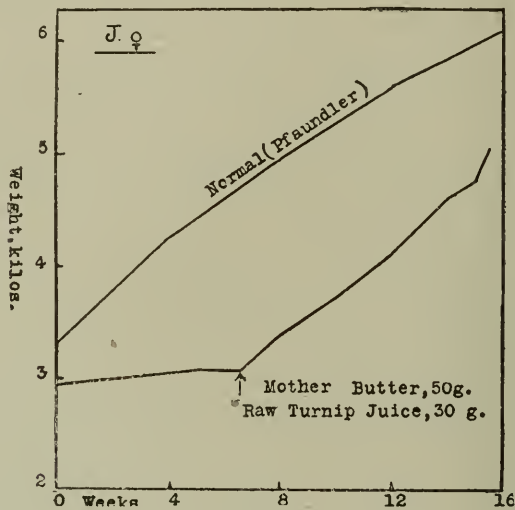


CHART 1.

was begun the child was 6 weeks old, and had put on only 100 grams in weight since birth. There was no increase in the amount of milk secreted by the mother.

Chart 2.—A weight chart showing similar improvement after addition of 20 grams of cod-liver oil daily to the mother's diet. In this case an increase in the mother's milk presumably caused an increase in calories in the child's food. Treatment began when the child was 5 weeks old and weighed less than 3 kilos, having put on no weight since birth. At 22 weeks, after seventeen weeks' treatment, including also some direct administration of cod-liver oil to the child, it weighed 5.7 kilos, the normal weight for this age being about 6.2 kilos.

Chart 3.—The weight chart of an artificially-fed infant of 6 months weighing 3 kilos (4 kilos below the normal), showing great improvement after addition to the diet of 10 grams raw turnip juice and 10 grams (later increased to 20 grams) of butter daily. In this case, owing to other

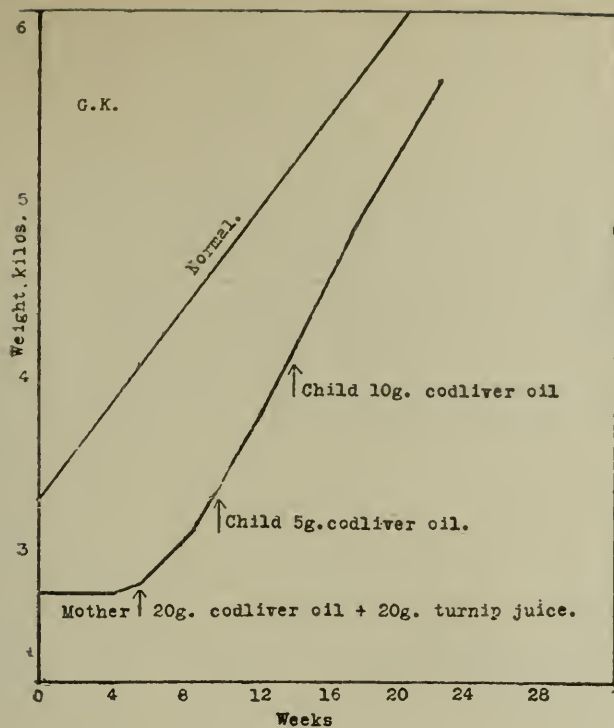


CHART 2.

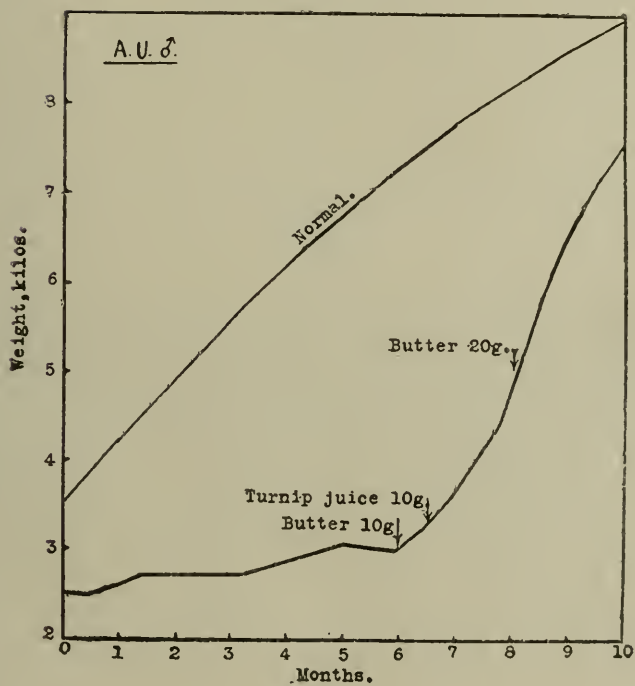


CHART 3.

changes in the diet, the child was receiving actually less calories after, than before, the change. After four months of treatment the child weighed $7\frac{1}{2}$ kilos, and was only 2 kilos below the normal for its age.

Chart 4 shows the weight curves of twins who at 8 months were much below normal development and weighed only 4.2 kilos and 3.5 kilos respectively. Both children had been receiving a diet deficient in milk fat. They improved immediately after addition to the diet of butter and cod-liver oil. The diet of the girl contained previously only 10 to 15 grams daily of milk fat. She was a miserable specimen, undersized, lethargic and pallid (haemoglobin 38 per cent.). At the point marked "Eye" on the curve she developed severe keratomalacia of the left eye, similar to that described by Bloch in Copenhagen. After five days, during which the symptoms became progressively worse in spite of local treatment, she received

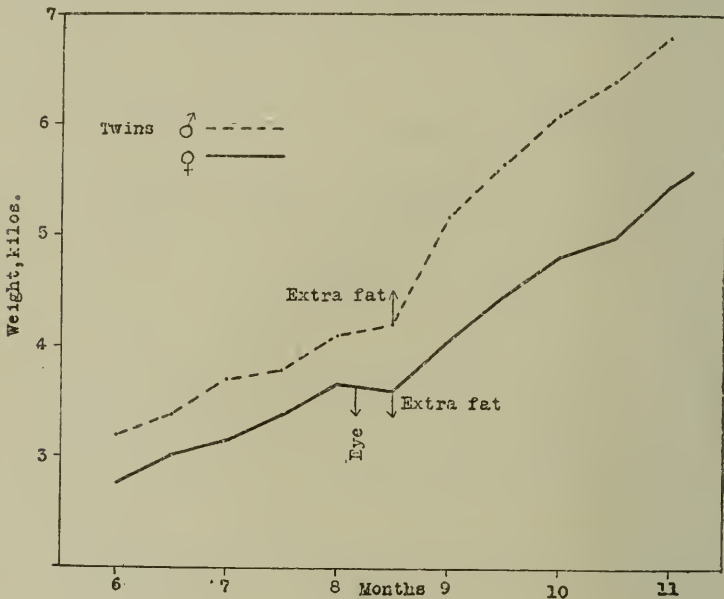
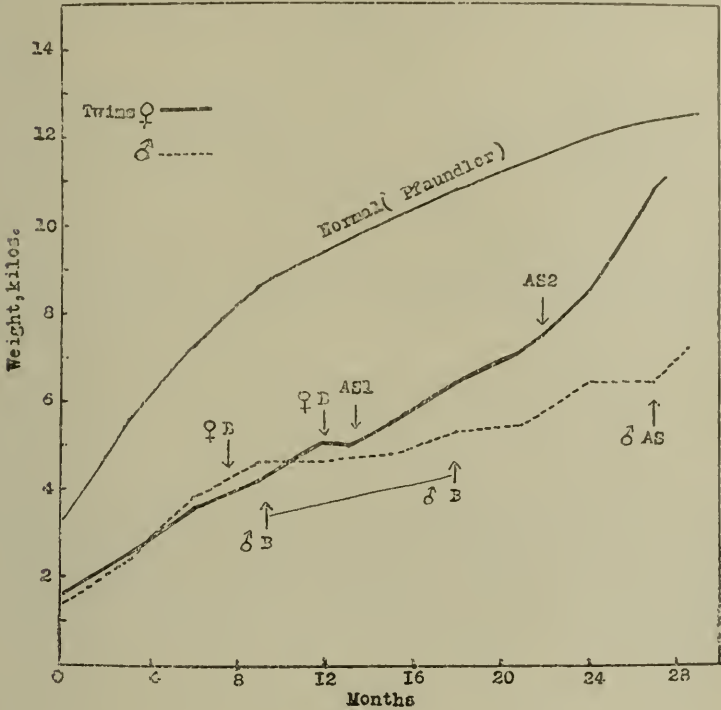


CHART 4.

10 grams of cod-liver oil emulsion and 20 grams of butter daily, raising her daily fat intake to 35 to 40 grams. She promptly began to improve and to put on weight; in fourteen days the eye was normal, and she had put on 300 grams in weight. She remained under observation for two and a half months, and at the end of the period was a jolly rosy infant weighing 5.6 kilos. The haemoglobin had risen to 70 per cent. At the time the treatment was begun the condition of the boy was rather less miserable. He weighed a little more than the girl, and the fat deprivation in his diet had been less severe. There were no eye symptoms. His general improvement after his diet had been adjusted was, if anything, more striking. After two and a half months he had put on 2.8 kilograms and weighed 6.8 kilos. The anaemia had greatly improved. In both cases the additions to the diet involved an increase in calories.

Chart 5.—The weight curves of twins of $2\frac{1}{2}$ years showing the favourable condition of the girl who had received abundant antiscorbutic and fat-soluble vitamine additions to diet during six months, compared with the ill-developed and rachitic state of the boy whose diet had not been so enriched. (This was further illustrated by photographs.) The treated child in this case showed, in addition to the actual increase in weight, the great improvement in general development, intelligence, muscular tone, and texture of the skin which characterized all the previous cases. In this instance lack of antiscorbutic in the diet seems to have been the important factor in retarding growth. The girl had shown definite symptoms of Barlow's disease at 8 months and



· CHART 5.

again at 12 months (at points marked B. ♀ on the curve); at 13 months antiscorbutic material was given as raw milk and growth began. At 22 months (at AS 2) more intensive treatment was started by giving raw lemon juice or raw turnip juice, together with butter (10 grams, and later 20 grams), and at 26 months 10 grams cod-liver oil was given as well. The boy had shown definite scurvy at 9 months, and the symptoms had persisted until 18 months, treatment with raw milk proving slow and ineffective in clearing up the condition; during this period of nine months the child had put on only 1 kilo in weight. The upward kick in his weight-curve at the 27th month corresponded to the addition of lemon juice to the diet.

Chart 6 gives the weight chart of a little girl who at 14 months weighed only 5 kilos. The rate of growth had become progressively slower since birth, and from the 6th to the 14th month the increase was only 500 grams. At this point symptoms of scurvy were apparent, and anti-scorbutic was introduced into the diet as raw milk.

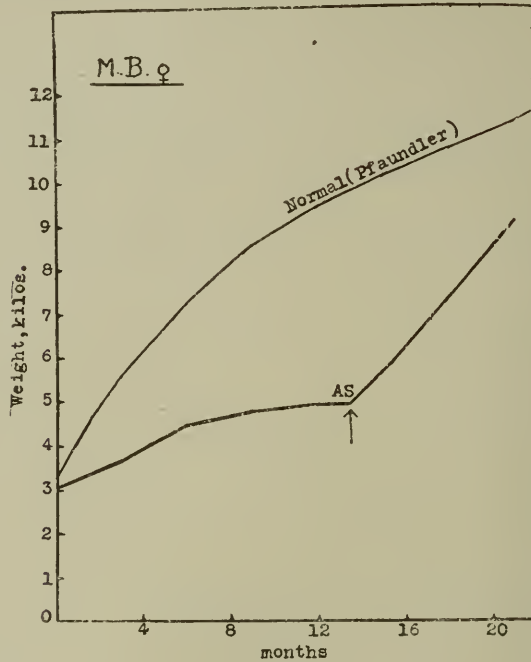


CHART 6.

An immediate improvement took place, indicating that the delay in growth during the previous months was to be attributed to lack of antiscorbutic. Raw milk was continued till the 17th month, when the child had put on nearly 2 kilos, and was sufficiently advanced to take a mixed diet.

THE INFLUENCE OF OVERCOOKING VEGETABLES IN CAUSING SCURVY AMONG CHILDREN.*

BY

HARRIETTE CHICK, AND ELSIE J. DALYELL,
LISTER INSTITUTE, BEIT MEMORIAL FELLOW.

(Report to the Lister Institute and to the Medical Research Council,
London.)

In April, 1919, 40 cases of scurvy occurred among the 64 children from 6 to 14 years of age under treatment for tuberculosis in the University Kinderklinik, Vienna (Professor C. v. Pirquet).

The first case, observed on April 12th, had pain in the jaws, swollen, tender, and discoloured gums, and later swollen and tender knees. On April 14th two more cases were reported and developed symptoms of the same severity. Subsequently 37 fresh cases occurred, the last on April 28th; in all of these the symptoms were mild and confined to gums and jaws.

From April 18th to April 28th 45 lemons were provided daily for the 64 children in the section, and extra spinach, "Kraut," and raw onions were added to the diet. The scorbutic patients received also a preparation made by boiling pine-tree needles in water. After April 28th extra supplies of fresh vegetables were obtained. In all cases the symptoms subsided in ten days, and in most in four or five days. In the majority of cases the weight charts show a temporary arrest in growth during the attack, but in no instance was there any evidence of a prolonged retardation.

The admirable arrangements of this hospital render the outbreak exceptionally favourable for study. A very accurate system of calculating food requirements has been elaborated by Professor v. Pirquet, and, as far as calories are concerned, each child has always received enough without any excess. The unusually complete records of diet which are kept as a routine measure permit of detailed analysis, and the average amount of each article of diet taken by each child can be accurately determined. Singularly full records are also kept of the growth in size and weight and the general condition of the patients, all of which were placed freely at our disposal by Professor v. Pirquet and his staff.

* A more detailed account of this investigation is being published in the *Zeitschrift für Kinderheilkunde*

At the time of the outbreak there was no corresponding prevalence of scurvy in the general population, and the conditions which determined its onset have to be looked for inside the hospital and primarily in the food.

Analysis of Diet.

The articles of food consumed in the Tuberculosis Section of the Kinderkliniek have been classified into:

(1) Those known to possess slight value or none for prevention of scurvy—flour, oats, barley, eggs, cheese, condensed milk, lard and other fat, dry beans and peas.

(2) Those possessing marked antiscorbutic properties—green vegetables, potatoes, other root vegetables, fresh meat, and fresh milk.

The comparative values of the foodstuff under (2) are given in Table I below. The figures are based on quantitative experimental work carried out at the Lister Institute on guinea-pigs and monkeys.¹ In many cases the

TABLE I.—*Showing Comparative Antiscorbutic Value of Equivalent Weights.*

Fresh lemon or orange juice, raw	100
Fresh cabbage leaves or expressed juice, raw	100
Fresh cabbage leaves, cooked at 100° C. for twenty minutes	30
Fresh cabbage leaves, heated 70° to 80° C. for sixty minutes	10
Fresh swede turnip juice, raw	60
Fresh tomatoes, raw	60
Fresh green beans, raw	30
Potato, cooked at 100° C. for thirty minutes	7.5
Fresh carrot juice, raw	7.5
Fresh beetroot juice, raw	...	less than	7.5
Fresh beef juice, raw	...	less than	7.5
Fresh cow's milk, raw	1 to 1.5
Dry beans, peas, lentils, etc., raw	...	less than	7.5
Germinated beans, peas, lentils, etc., raw	30

values have been confirmed by recent therapeutic trials in Vienna on infantile scurvy (Barlow's disease). The values of the fresh materials are, of course, much diminished by the ordinary procedure of cookery.

The possible influence of meat has been disregarded in the present investigation. Even when raw its antiscorbutic value is small and it must be consumed in very large amounts—for example, 500 grams and upwards per person daily²—if scurvy is to be prevented by its agency alone. During the six months preceding this outbreak the weekly average per patient was less than 300 grams. We do not consider either that fresh milk has played any important part as an antiscorbutic in the present instance. At the best its antiscorbutic value is low, and owing to disorganized transport it was almost certainly heated twice before reaching the children. Up to the end of January, 1919, the average daily ration was never more than 250 c.cm., and during February and March this steadily declined to about 30 c.cm. More condensed milk was then used, but the value of this product is even less than that of fresh unheated milk. No fruit was taken. The average consumption of green vegetables, potatoes, and other root

vegetables is given in Table II. The outstanding feature of this analysis is the deprivation of the most effective antiscorbutics after the middle of February. During the autumn of 1918 there appears to have been a generous provision of green vegetables, potatoes, etc. This was on the whole maintained during January, 1919, but there was a great falling off in February, which became still more marked in March.

These data appear to show clearly that a daily ration of about 80 grams root vegetables is insufficient to protect from scurvy, and we are of opinion that the marked shortage of all vegetables during the eight weeks from the middle of February to the middle of April was the immediate precipitating cause of the outbreak. If, however, this were the whole explanation, it is remarkable that such a short period of deprivation should cause widespread recognizable scurvy. For adults the necessary period has

TABLE II.—*Average Daily Consumption by each Patient in Grams.*

Date.	Green Vegetables.	Potatoes.	Other Root Vegetables.	Total Vegetables.	Milk.	
					Fresh.	Con-densed.
1918.						
September-October ...	102	114	15	231	188	30
November-December .	62	110	62	234	225	16
1919.						
January	30	130	68	228	190	112
February (first half) ...	0	55	92	147	33	215
February (second half)	0	0	101	101		
March	0	0	77	77	28	105
April (first fortnight) .	12	1	53	66	0	80

been fixed at from four to eight months, and oftener perhaps nearer the longer time.³ For children there appears to be no definite information beyond the fact that symptoms are rarely seen in infants less than 6 months old.⁴ It is not unlikely that the period for children is less than that for adults in correspondence with their greater metabolism, but there do not seem to be any grounds for taking it as shorter than four months.

In the present instance the allowance of vegetables up to two months before the first cases appeared was more than 200 grams daily, which is not less than is usual in many private households during the winter, and may be expected to prove adequate for prevention.⁵ A similar allowance, 250 grams daily fresh vegetables, is a common ration for soldiers on active service, and instances must be numerous in which this amount has failed temporarily without appearance of scurvy in so short a period as eight

weeks. In the present case, therefore, the antiscorbutic taken in the previous months should have enabled these children to withstand eight weeks of comparative deficiency. It did not do so, and we are forced to conclude that while the supply of vegetables was adequate the supply of antiscorbutic was defective.

The explanation seems to lie in the method of cooking; this process destroys the antiscorbutic vitamine in proportion to the time and temperature of heating. The time of cooking is in general more important than the temperature, and it has been shown that quick heating at 100° C. entails less destruction than prolonged simmering at 70° to 90° C.⁶ Outbreaks of scurvy in which excessive cooking of the vegetable ration has been considered an important contributory cause have been already placed on record.⁷

By the kindness of Professor v. Pirquet, and with the help of the sister in charge of the kitchen, we were enabled to study the methods of cooking employed in the kitchen of the Kinderklinik.

In cooking for institutions, where large numbers of people are concerned and large quantities of food have to be manipulated, the time taken in cooking must of necessity be much longer than is the case in small households. In Vienna, moreover, the usual method of preparing vegetables involves two separate cooking processes: they are first boiled till soft, and afterwards cooked again before serving, in an "Einbrenn" made from flour and fat. This double cooking involves a twofold destruction of antiscorbutic vitamine.

Similar methods of cooking vegetables have been employed in the kitchen of the Kinderklinik. Even potatoes are frequently twice cooked—first boiled or cooked in steam at 100°C., then sliced and cooked again with fried fat and onions. In the making of soups the root vegetables, tomatoes, green vegetables, etc., are subjected to a temperature of 90° to 100° C. for as long as three to four hours as a general rule. Following such methods of cooking, the loss of antiscorbutic value must be very extensive, if not almost complete. It is probable, therefore, that during the period (September, 1918, to February, 1919) when an adequate amount of fresh vegetables was provided, the diet nevertheless was a scurvy-producing one, due to loss of antiscorbutic vitamine during the cooking.

It would be easy to introduce slight modifications into the method of cooking which would avoid much loss of antiscorbutic material and would not alter seriously the present manner of preparing the food. The following are examples:

1. *Soups*.—In the making of soups the vegetables are first cut in small pieces, placed in water, and the whole cooked together for three or four hours. If green vegetables are used, or fresh tomatoes, as in "Kohl-suppe" or "Paradeis-suppe," these also are subjected to the whole period of cooking. An improved method would be to add the sliced cabbage or the tomatoes, etc., shortly before serving the soup, so that the cooking should take

place for as short a time as possible. When fresh fruit and vegetables are scarce it would be an excellent plan to grate the root vegetables and press out the juice, and to boil the dry pulp with the other constituents of the soup for as long a time as is needed, reserving the raw juices to be added just before the soup is served.

2. *Green vegetables* [Kochsalat (cos-lettuce); Kohl (cabbage), etc.] are washed, placed in hot water, heated to boiling point and kept at 90 to 100° C. until soft (about 1½ hours). The water is then poured off, and only a little retained to add to the finished dish. The cooked greens are minced, heated a second time with an "Einbrenn" made of flour, bacon fat, onion, etc., water being added to make the right consistency. It would be better to steam the green leaves instead of cooking them in boiling water. The time of cooking could probably be shortened to twenty to thirty minutes, and the loss of antiscorbutic and other vitamins in the water (which is not consumed) would be avoided.

Germinated Seeds.—The use of germinated peas, beans, lentils, or other seeds as supplementary antiscorbutic materials where there is a shortage of fresh vegetables cannot be too strongly urged.

The method of preparation is as follows: The beans, peas, etc., are washed in water and left to soak for twenty-four hours at room temperature. They are placed in layers not exceeding 5 to 7 cm. in depth upon an ordinary kitchen sieve or other porous surface. They are kept moist by occasional sprinkling with water and with free access of air for 48 to 72 hours. At the end of this period rootlets varying from 0.5 to 1 cm. in length should be visible. The germinated seeds should not be allowed to become dry again, but should be cooked immediately. The cooking should be for as short a time as is necessary to render them soft and palatable. Placing in water for half an hour and then boiling for ten to fifteen minutes should suffice for small seeds such as peas and beans.

The antiscorbutic value of this germinated material has been determined by means of experiments with animals (guinea-pigs) and has been found, weight for weight, to possess about one-third the value of cabbage leaves in the raw condition.⁸ It has also been successfully used in the prevention and cure of human scurvy.⁹ It is interesting to note that dry peas, beans, and "czirok"* formed an important article of diet in the tuberculosis ward during the whole period under investigation. In the three months previous to the outbreak an average amount of 33.5 grams were consumed daily by each child. During the time of greatest dearth—that is, the eight weeks immediately preceding the outbreak—a daily average of 30 grams per head was consumed. When dry these pulses have a negligible antiscorbutic value, but had they been given in the germinated condition the ration of 30 grams would have represented the equivalent of about 60 grams of fresh vegetables, if not spoilt by overcooking. This extra allowance would have been of inestimable value in preventing the outbreak.

The incidence of scurvy in relation to the length of residence in hospital is of some interest, the children who

* A sort of buckwheat.

had been in hospital for a longer period being affected a good deal more than the recent arrivals.

TABLE III.

Group.	Residence in Hospital.	Number.	Scurvy Symptoms.	Per Cent.
A	6 months or more ...	17	16	94
B	3 to 6 months ...	21	12	57
C	Less than 3 months ...	26	12	46

It has been pointed out above that the children in Group A had probably suffered long relative deprivation of antiscorbutic owing to the methods used in cooking their vegetables. The same conditions did not apply to Groups B and C, though it is certain that these children suffered deprivation in their homes before entering hospital, for the whole city was reduced to a food supply meagre in quantity and of the poorest quality during the winter 1918-1919, and fresh food was specially scanty. It is, indeed, remarkable that the conditions as regards scurvy were apparently less satisfactory in hospital than among the general population. Among the latter there was certainly no general outbreak of scurvy, and the children who had been but a short time in hospital were less disposed to develop the disease when the acute shortage of antiscorbutic came than those who had been in longer. The presumption is that during the six months or so preceding the outbreak the general population were getting more antiscorbutic than these hospital patients.

The possibility that admission to hospital precipitated the onset of the disease in Groups B and C is also worth consideration. There is considerable evidence that the demand of the body for antiscorbutic is in proportion to the rate of metabolism. In Group A the weight charts are very uniform and normal for seven months before the outbreak and show a normal rate of increase. Groups B and C, on the other hand, are composed of children who on admission were much below the normal age weight owing to general underfeeding. The diet of the Klinik, ample in calories, led to abnormally rapid growth, and this may well have had something to do with the onset of scurvy.

SUMMARY.

1. An outbreak of scurvy in April, 1919, affected forty children out of sixty-four aged between 6 and 14 years under treatment for tuberculosis.

2. From September, 1918, to February, 1919, a good supply of fresh vegetables and milk was available. From the middle of February to the middle of April the daily ration of fresh vegetables was reduced to about 80 grams per head; deprivation was never complete.

3. The eight weeks' period of shortage preceding the outbreak is a much shorter period than the four to six months

noted by other observers as necessary for the development of scurvy.

4. It is concluded that though the supply of fresh food was adequate, the diet was nevertheless a scurvy producing one, and that the period of deprivation of antiscorbutic was longer than it appeared to be.

5. An explanation is found in the method for cooking food employed in the hospital which involves a serious destruction of antiscorbutic. Suggestions are made as to means of preventing this.

6. The germination of dry peas, beans, and lentils before cooking is strongly recommended as a means of providing extra antiscorbutic when fresh vegetables and fruit are scarce or absent.

7. It is suggested that abnormally rapid growth may promote the development of scurvy in children.

In conclusion, we desire to express our gratitude to Professor v. Pirquet, who placed all his valuable records freely at our disposal, and to the members of his nursing staff who assisted us in every way in our investigations.

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(Aus der Universitäts-Kinderklinik in Wien [Vorstand: Prof. Dr. C. Pirquet].)

Eine Skorbutepidemie unter Kindern im Alter von 6 bis 14 Jahren.

Von

Harriette Chick, London (Lister-Institut)

und

Elsie J. Dalyell, Sydney, Australia (Brit. Memorial Fellow).

(Bericht an das Lister-Institut und den Medical Research Council, London.)

Mit 1 Textabbildung.

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Im April 1919 wurden unter 64 Patienten der Tuberkuloseabteilung der Universitätskinderklinik in Wien 40 Fälle von Skorbut beobachtet. Um die Ursache des damaligen plötzlichen Auftretens dieser Krankheit zu erforschen, ist eine gründliche Untersuchung der Vorgeschichte der einzelnen Fälle sowie der Menge und Art der Ernährung in den der Erkrankung vorhergehenden Monaten mit Hilfe von Tabellen und statistischem Material vorgenommen worden.

Zur Zeit des ersten Auftretens der Krankheit (April 1919) befanden sich 64 Kinder in der Abteilung. Die Beziehung zwischen Dauer des Spitalaufenthaltes und Erkrankung am Skorbut zeigt folgende Tabelle:

Gruppe	Aufenthaltsdauer im Spital	Zahl	Skorbutsymptome beobachtet	Keine Skorbutanzeichen
A	6 Monate oder länger	17	16	1
B	3—6 Monate	21	12	9
C	Weniger als 3 Monate	26	12	14
Summa		64	40	24

Von den 40 skorbutkranken Kindern standen 16 im Alter von 10 bis 14 Jahren, 24 in dem von 6—10 Jahren. Von den letzteren befanden sich 12 in Gruppe A, d. h. sie waren schon seit mehr als 6 Monaten im Spital.

Der erste Fall wurde am 12. IV. 1919 beobachtet; der Patient hatte Schmerzen im Ober- und Unterkiefer, das Zahnfleisch war dunkel gefärbt, geschwollen und empfindlich, und weiterhin zeigten sich auch

die Knie geschwollen und schmerzhaft. Am 14. IV. kamen 2 neue Fälle zur Anzeige und zeigten Symptome von gleicher Schwere. Im weiteren Verlauf traten noch 37 Fälle auf, der letzte am 28. IV.; bei diesen allen waren die Symptome milder und beschränkten sich auf Kiefer und Zahnfleisch.

Vom 18. IV. an fand eine allgemeine antiskorbutische Behandlung für die ganze Abteilung statt, woraus sich die durchgehend milderen Formen der weiteren Erkrankungen erklären lassen.

Behandlung. Vom 18. IV. bis zum 28. IV. wurden täglich 45 Citronen für die 64 Kinder in der Tuberkuloseabteilung gebraucht und Extraportionen von Spinat, Kraut und rohen Zwiebeln in die Kost aufgenommen. Die an Skorbut erkrankten Kinder bekamen außerdem einen Aufguß von Tannennadeln [siehe Tobler¹⁸]. Nach dem 28. IV. konnten noch größere Mengen frischer Gemüse beschafft und in die Kost noch reichlicher aufgenommen werden.

In allen Fällen schwanden die Skorbutsymptome sehr rasch; die am schwersten Erkrankten zeigten schon nach 10 Tagen keine Anzeichen mehr, und in der Mehrzahl der Fälle waren die Symptome nur 4—5 Tage erkennbar. Hierzu ist zu bemerken, daß das Personal der Klinik schon die leisesten Anzeichen einer Erkrankung bemerkte und zur Anzeige brachte.

Die Gewichtstabellen derjenigen Kranken, die im Spital verblieben, zeigten in der Mehrzahl der Fälle einen Stillstand in der Gewichtszunahme während der Krankheit, aber auch wieder rasche Rückkehr zum normalen Tempo. Keine einzige Tabelle zeigt eine anhaltende Verzögerung dieses Tempos nach Erkrankung am Skorbut. Das Studium der Gewichtstabellen sämtlicher Skorbutfälle hat folgende interessante Resultate ergeben.

In Gruppe A sind die Gewichtskurven während einer Periode von 7 Monaten ziemlich gleichförmig; sie zeigen unbedeutende Änderungen in den Wochenzahlen der Gewichte und eine durchschnittliche Zunahme, die sich der Normalzunahme von 2—3 kg im Jahr annähert. In Abb. 1 sind die Kurven 1, 2, 3 die der Patienten (alle 3 der Gruppe A zugehörig), die zuerst die Skorbuterscheinungen zeigten. Die Kurven 8—13 wurden Fällen aus den Gruppen B und C entnommen. Bei diesen ist das Gewicht bei der Aufnahme ins Spital tief unter dem für das Alter der Patienten normalen, und vorerst erfolgte eine schnelle Zunahme, bis der normale Stand erreicht oder überschritten ist, worauf die Gewichtszunahme ein gleichmäßiges, normales Tempo annimmt. In der Universitätskinderklinik kommt bekanntlich das Pirquetsche Nemsystem der Ernährung in Anwendung, das eine sehr genaue Berechnung des Nahrungsbedarfes enthält; dementsprechend wird die notwendige Calorienmenge immer voll verabreicht, aber nicht über-

schritten. Die Erfahrung hat gelehrt, daß die Patienten der Tuberkuloseabteilung sehr schnell auf diese Ernährungsweise reagieren, indem sie sofort nach der Aufnahme außergewöhnlich rasch an Gewicht zunehmen.

Besonders auffallend war dies bei den Kindern, die im Winter 1918/19 Aufnahme fanden, nachdem sie unter den Folgen der damals in Wien herrschenden Hungersnot gelitten hatten. Diese Beobachtung ist für diejenigen Kinder wichtig, die am Skorbut erkrankten schon nach einem Spitalsaufenthalt, der kürzer war als die Frist, die in der Regel notwendig ist, damit Skorbut sich entwickle. Aus der Besprechung der Lebensbedingungen der Gruppe A, die weiter unten erfolgt, wird hervorgehen, daß für diese Kinder schon während des Spitalsaufenthaltes in bezug auf antiskorbutische Substanz eine Mangelperiode von mehreren Monaten bestanden hat, die dazu hinreichte, um die Entwicklung von Skorbut zu begünstigen. Diese Vorbedingungen können jedoch bei Gruppe B und Gruppe C nicht als zutreffend angesehen werden, da hier der Spitalsaufenthalt kürzer ist. Es ist wohl sicher, daß auch diese Kinder zu Hause vor dem Eintritt ins Spital schlecht ernährt waren, denn die ganze Stadt war während des Winters 1918/19 auf eine Lebensmittelmenge von äußerster Knappheit und schlechter Qualität beschränkt, wobei insbesondere frische Lebensmittel nahezu ganz fehlten. Trotzdem wurde gehäuftes Auftreten von

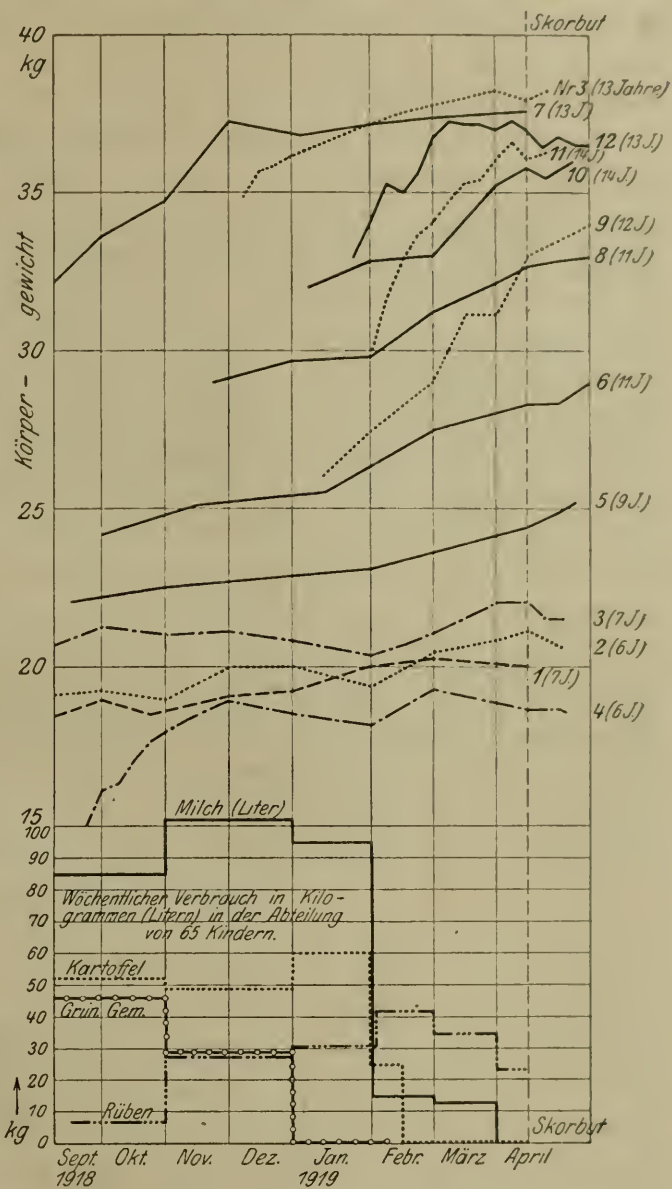


Abb. 1.

Skorbut bei der Bevölkerung nirgends beobachtet, so daß die Annahme nicht gestattet erscheint, als wären vorhergehende Entbehrungen der einzige Hilfsfaktor gewesen, der zu der Erwerbung des Skorbut durch die Kinder innerhalb von 2—3 Monaten nach ihrer Aufnahme ins Spital beigetragen hätte. Es ist hingegen sehr wahrscheinlich, daß bei diesen Fällen als zweiter Faktor die Steigerung des Stoffwechsels hinzukommt, die während des durch die Spitalsbehandlung verursachten anfänglichen rapiden Wachstums eintreten mußte.

Es gibt nämlich gewichtige Beobachtungen, die darauf hindeuten, daß der Körperbedarf an antiskorbutischen Vitaminen vom Stoffwechsel abhängt. Verschiedene Berichte über das Auftreten von Skorbut zeigen, wie sehr durch eine außergewöhnliche Beschleunigung des Stoffwechsels bei unzureichender Ernährung das Auftreten der Krankheit beeinflußt werden kann. Man hat beobachtet, daß auf langen Seereisen die Matrosen, die der Kälte stark ausgesetzt waren, früher an Skorbut erkrankten als solche, die weniger exponiert waren [Lind, 1747¹⁶]; daß während der Belagerung einer Stadt die Soldaten, die schweren Dienst machten, der Krankheit eher zum Opfer fielen als die Einwohner (Lind); daß bei arktischen Expeditionen die Krankheit ohne jedes vorherige Anzeichen plötzlich auftrat, sobald mit dem anstrengenden Ziehen der Schlitten begonnen wurde¹⁷); und endlich zeigten sich auch in jüngster Zeit in einem Gefangenenlager die ersten und schwersten Fälle in einer Abteilung, wo schwere körperliche Arbeit geleistet wurde¹⁸) (S. 67). Wir neigen daher der Ansicht zu, daß das schnelle Wachstum der Kinder der Gruppen B und C die Entwicklung der Krankheit während einer, wenn auch kurzen Zeit des Mangels an frischen Nahrungsmitteln beschleunigt hat.

Zusammensetzung der Kost vom September 1918 bis Februar 1919.

Die genauen Angaben über die Kost, wie sie an dieser Station verabreicht wurde, sind uns von Herrn Prof. Pirquet und den an seiner Klinik Beschäftigten mit großem Entgegenkommen zur Verfügung gestellt worden. Die außerordentlich vollständigen Aufzeichnungen über die Diät, auf die an dieser Klinik streng gesehen wird, gestatten einen genauen Einblick in die Zusammensetzung der Nahrung bis ins einzelne und die sichere Feststellung der Durchschnittsmenge an jedem Kostbestandteil, die das einzelne Kind genossen hat.

Die in der Tuberkuloseabteilung verwendeten Nahrungsmittel lassen sich folgendermaßen einteilen:

1. Solche, die erfahrungsgemäß geringen oder gar keinen skorbutverhütenden Wert besitzen.
2. Solche, die ausgesprochene skorbutverhütende Wirkung haben.

1. Nahrungsmittel von geringem oder fehlendem antiskorbutischen Wert	2. Nahrungsmittel von skorbutverhütendem Wert
Mehl	Blattgemüse
Eier	Kartoffeln
Haferreis	Rüben
Rollgerste	Frische Milch
Fett	Fleisch
Kondensmilch	
Käse	
Bohnen } in trockenem Zu-	
Erbsen } stande	

Diese beiläufige Einteilung gründet sich auf die Ergebnisse neuerer experimenteller Arbeiten im Verein mit Beobachtungen über Ernährung anlässlich klinischer Skorbutstudien. *)

Die folgende Tabelle I ist einer früheren Arbeit der Verfasserinnen entnommen⁶⁾ und zeigt die Vergleichszahlen für den skorbutverhütenden Wert verschiedener Nahrungsmittel, wie er durch eine am Lister-Institut London ausgeführte quantitative Experimentalarbeit mit Meerschweinchen und Affen ermittelt wurde. In jüngster Zeit hat

Tabelle I.

Vergleichszahlen für den skorbutverhütenden Wert äquivalenter Gewichte verschiedener Nahrungsmittel.

Material	Vergleichszahl	Quellenangabe
Frischer Citronensaft	100	9)
Frischer Orangensaft	100	9)
Frische Kraut- od. Kohlblätter (od. ausgedr. Saft), roh	100	11)
Frischer roher Saft von schwedischen Rüben (Wruken, Schmalzrüben usw.) (Genus Brassica).	60	10)
Frischer Tomatensaft, roh	60	**)
Frische grüne Bohnen	30	4)
Frische gekeimte Hülsenfrüchte, roh	30	5)
Frischer Mohrrüben- (Karotten-) Saft, roh	7,5	10)
Frischer Saft von Salatrüben, roten Rüben, Runkelrüben (Genus Beta), roh	7,5	10)
Frischer Rindfleischsaft, roh	< 7,5	8)
Kartoffeln, 30 Min. auf 100° C erhitzt	< 7,5	10)
Frische Kuhmilch, roh	1—1,5	3. 7)
Trockene Hülsenfrüchte, roh	< 7,5	5)

*) Eine mit genauem Literaturverzeichnis versehene Besprechung dieses Gegenstandes findet sich in dem „Report on the Present State of Knowledge concerning Accessory Food Factors“. Medical Research Committee. Special Report Series Nr. 38. H. M. Stationery Office, London 1919.

**) Nicht veröffentlicht.

man in Wien gewisse von diesen Nährstoffen versuchsweise zur Heilung von Kinderskorbut (Barlowsche Krankheit) angewendet. Die Ergebnisse dieser Beobachtungen haben im allgemeinen die experimentell gefundenen Wertziffern bestätigt und sollen in einer späteren Publikation beschrieben werden.

Fleisch und Milch. In dieser Untersuchung ist die Wirksamkeit des Fleisches als skorbutverhütender Nährstoff aus folgenden Gründen vernachlässigt worden. Vor allem hat frisches Fleisch selbst in rohem Zustande im Vergleich mit vielen frischen Gemüsen eine niedrige antiskorbutische Wertigkeit. In den Fällen, wo Skorbut durch die alleinige Wirksamkeit des Fleisches in der Kost verhütet wurde, waren erfahrungsgemäß große Mengen davon notwendig (z. B. ein täglicher Verbrauch pro Person von 500 g aufwärts), Curran (1847)¹¹), Hehir (1917)¹⁴), Stevenson (1919) (unveröffentlichte Beobachtungen). Die für diese Zahl von 65 Kindern verwendete Wochenmenge an Fleisch überstieg in den 3 Monaten unmittelbar vor Ausbruch der Krankheit nie das Gewicht von 15 kg, und in den vorausgehenden 6 Monaten nie das von 20 kg, was eine wöchentliche Durchschnittsmenge pro Person von weniger als 300 g ergibt.

Milch. Wir sind auch nicht der Ansicht, daß in dem vorliegenden Falle frische Milch als Antiskorbutikum eine bedeutende Rolle gespielt hat. Eine durchschnittliche Wochenmenge von ca. 100 l konnte bis Ende Januar beschafft werden; diese sank auf 15 bzw. 13 l im Februar und März. Wie aber aus Tab. I hervorgeht, bleibt die skorbutverhütende Wertigkeit selbst frischer, roher Milch weit hinter der einer entsprechenden Menge von Blattgemüse oder Rüben zurück. Dazu kommt noch, daß bei den schlechten Transportverhältnissen die frische Milch sicherlich vor dem Verbrauch zweimal erhitzt wurde und daher jedenfalls einen sehr herabgesetzten, wenn überhaupt einen antiskorbutischen Wert besessen hat. Die von dem einzelnen Kinde genossene tägliche Menge überstieg nie einen Viertelliter und verminderte sich stetig während des in Frage stehenden Zeitabschnittes. Zugleich stieg allerdings der Verbrauch an Kondensmilch, deren Wertigkeit aber bedeutend tiefer stehen soll als die der frischen Milch. Wir können daher nicht glauben, daß die Milch in dieser Zubereitung in so geringen Mengen eine irgendwie ausschlaggebende Bedeutung als Antiskorbutikum haben konnte.

Von sonstigen antiskorbutisch wirksamen Nahrungsmitteln wurden an der Abteilung verwendet: Blattgemüse, Kartoffeln und Rüben. Auch die wöchentlich für alle Kinder verbrauchte Durchschnittsmenge an diesen Nährstoffen wurde berechnet, und die betreffenden Zahlen sind aus Tab. II ersichtlich, ebenso wie die tägliche Durchschnittsmenge für das einzelne Kind.

Tabelle II.

Wöchentlicher Durchschnittsverbrauch an skorbutverhütenden Substanzen von 65 Patienten während der dem Auftreten des Skorbutus vorausgehenden 6 Monate, in kg ausgedrückt						Täglicher Durchschnittsverbrauch der einzelnen Patienten, in Gramm ausgedrückt			
Zeit	Blatt- gemüse	Kar- toffeln	Rüben	Milch		Gemüse	Milch		
				frisch	kond. *)		frisch	kond. *)	Zusammen
Sept./Okt. 1918	46,5	52	7	85,5	13,7	230	188	30	218
Nov./Dez. 1918	29	49,5	29	102	7,2	240	225	16	241
Januar 1919	14	59,5	31	95,5	51	225	190	112	302
Februar 1919	0	(erste 2 Woch. 25; später 0)	42	15	99	(erste 2 Woch. 140; später 71)	33	215	248
März 1919	0	0	35	13	48	74	28	105	133
April 1919 (die erst. 2 Woch.)	5,6	0,6	24	0	35	66	0	80	80

Die hervorstechendste Tatsache dieser Zusammenstellung ist der Mangel an skorbutverhütendem Stoff nach dem 15. II. 1919, mit Ausnahme der Rüben. Während des Herbstes 1918 scheint die Versorgung mit grünem Gemüse, Kartoffeln, Rüben noch recht ausgiebig, und dies hielt im großen ganzen bis Ende Januar 1919 an, aber im Februar trat ein großer Sturz ein, der sich im März noch verschlimmert.

Was das Blattgemüse anbetrifft, betrug der durchschnittliche Wochenverbrauch z. B. im November und Dezember 1918 29 kg, im Januar sank er auf 14 kg, um im Februar und März ganz zu verschwinden. Die Kartoffelration wird im Januar im gleichen Ausmaße aufrechterhalten wie in den vorhergehenden Monaten, eine kleinere Menge konnte noch bis zum 15. II. aufgebracht werden, mit Ende dieses Monats verschwindet diese Knolle aus der Kost. Der Verbrauch an Rüben blieb im Zeitabschnitt November 1918 bis April 1919 im allgemeinen der gleiche, und zwar wurden wöchentlich zwischen 30 und 40 kg davon für die 65 Patienten aufgewendet.

Ein Blick auf die siebente Rubrik der Tab. II zeigt, daß bis Ende Januar die auf den einzelnen entfallende Menge an frischem Gemüse eine ausgiebige war, da sie sich auf 200 g täglich belief, noch während der zwei ersten Februarwochen auf 170 g pro Kind. Vom 14. II. bis zum 12. IV. (an welchem Tage die ersten zwei Skorbutfälle entdeckt wurden) wurde die durchschnittliche tägliche Verbrauchsmenge auf 70 g herabgesetzt und enthielt kein Blattgemüse mehr. Dieser Zeitraum achtwöchigen Mangels, der dem Ausbruch der Krankheit unmittelbar vorausging, ist viel kürzer als die sonst für die Entwicklung der Krankheit erfahrungsgemäß als notwendig festgestellte Zeitdauer.

*) Ausgedrückt entsprechend der Literzahl an frischer Milch.

Die Schnelligkeit, mit der ausgesprochene Skorbutsymptome sich zeigten, nachdem die Verbrauchsmenge an frischem Gemüse eingeschränkt worden war, deutet darauf hin:

1. daß eine durchschnittliche Tagesmenge von 70 g Rüben zur Verhütung von Skorbut nicht hinreicht;

2. daß noch ein anderer, skorbutfördernder Faktor am Werke sein mußte.

Bei den Kindern der Gruppen B und C wurde eine besondere Erklärung für die rasche Entwicklung der Krankheit in ihrer plötzlichen schnellen Gewichtszunahme gefunden. Für die Kinder der Gruppe A hat diese Erklärung keine Geltung, und die Aufmerksamkeit muß sich der Kost der letzten 6 Monate vor dem Auftreten der Krankheit näher zuwenden.

Zeitdauer der Entwicklung des Skorbut. Die Angaben über die Zeit, die verstreichen muß, damit sich beim Genuß einer skorbutbegünstigenden Kost deutlich erkennbare Skorbutsymptome entwickeln, schwanken bei verschiedenen Beobachtern zwischen 4 und 8 Monaten. In früheren Zeiten, als frische Nahrung im Winter immer selten war oder ganz fehlte, pflegte der Skorbut nach mehreren Monaten des Mangels im Frühjahr aufzutreten. Hehir (1917)¹⁴) stellt fest, daß 4 Monate die kürzeste Frist ist, in der nach seinen Beobachtungen der Skorbut sich bei im Heeresdienst stehenden indischen Truppen entwickelte. Holst und Fröhlich (1912)¹⁵) zitieren zwei gut geprüfte Fälle, bei denen die Entwicklungsdauer eine viel längere war, nämlich 6 bzw. 7½ Monate. Beim Säuglingskorbut (Barlowsche Krankheit) werden deutliche Anzeichen selten vor dem 7. Lebensmonat beobachtet [Barlow¹)].

Bei der von uns hier untersuchten Skorbutepidemie wurden die ersten deutlichen Symptome Mitte April 1919 beobachtet, und wir nehmen an, daß die Kost mindestens während der vorausgegangenen 4 Monate skorbutverhütender Stoffe entbehrte. Nun erhellt zwar aus den in Tab. II zusammengefaßten Erhebungen über die Kost, daß die antiskorbutischen Substanzen in derselben seit Mitte Februar, d. h. 8 Wochen vor dem Auftreten der Krankheit, stark eingeschränkt worden waren. Aber vor diesem Datum, nämlich während der ganzen dem Krankheitsausbruch vorausgehenden 6 Monate, ist ein solcher Mangel in der Kost nicht leicht zu erklären.

Es ist uns nicht gelungen, in der Literatur Angaben darüber zu finden, welche Menge an antiskorbutischen Nährstoffen notwendig wäre, um Kinder dieses Alters vor Skorbut zu bewahren. Die unten zitierten Beispiele beziehen sich auf erwachsene Männer, und wir führen sie zu Vergleichszwecken an. Der Verhältniswert der verschiedenen erwähnten Substanzen kann mit Hilfe der Tab. I berechnet werden.

Stevenson (unveröffentlichte Arbeit 1919) heilte den bei russischen Gefangenen bereits ausgebrochenen Skorbut durch eine täglich verabreichte Menge von 450 g gekeimter Bohnen; Wiltshire (1918)²⁰) fand sogar 225 g gekeimte Bohnen im Tag hinreichend, um bei serbischen Soldaten Heilerfolge zu erzielen. 225 g gekeimter Bohnen war auch die tägliche Ration, die bei englischen Truppen während des Winterfeldzuges 1918/19 verabreicht wurde und die, obwohl die Kost sonst keine frischen Nahrungsmittel enthielt, das Auftreten von Skorbut erfolgreich verhütete.

Die sonstigen verlässlichen Angaben über gemachte Beobachtungen beziehen sich meistens auf die Wirkung von Citronensaft. Die vorschriftsmäßige tägliche Dosis, die in der britischen Marine am Anfang des 19. Jahrhunderts zur Verhütung des Skorbutis diente, betrug nur $\frac{1}{2}$ Unze (15 g) (Budd, 1840)²⁾. Zweifellos ist die Tatsache, daß der Skorbut so gut wie ganz zum Verschwinden gebracht wurde, dieser Maßregel zuzuschreiben.

Im vorliegenden Falle bekam jedes Kind der Gruppe A von September bis Februar täglich im Durchschnitt mehr als 200 g frisches Gemüse. Ein solches Quantum ist nicht geringer als das in vielen Privathaushaltungen übliche und wäre eigentlich hinreichend, um Skorbut zu verhüten. Ein nicht viel größeres, 250 g frisches Gemüse täglich, ist für Soldaten im Frontdienst ein gewohntes Quantum, und es kommt daher sehr häufig vor, daß diese Menge zeitweilig fehlte, ohne daß es in der kurzen Zeit von 8 Wochen zum Auftreten von Skorbutssymptomen kam. Wenn auch von Mitte Februar angefangen die tägliche Zuzufuhr auf ungefähr 70 g frisches Gemüse pro Kopf herabsank, so hätte doch die in den vorausgegangenen Monaten eingenommene Menge an antiskorbutischem Material die Kinder dazu befähigen müssen, 8 Wochen (15. II. bis 12. IV.) verhältnismäßigen Mangels zu ertragen, ohne Skorbutssymptome zu zeigen. Der Schluß ist unvermeidlich, daß die Kinder, obwohl sie ein entsprechendes Quantum an frischen Gemüsen genossen hatten, doch nur mit einer unzureichenden Quantität antiskorbutischer Stoffe ausgestattet worden waren.

Kochmethode. Die Erklärung für diese Tatsache liegt anscheinend darin, daß die Gemüse nicht roh verzehrt wurden und sehr viel antiskorbutisch wirksame Substanzen während des Kochens verloren gingen. Der antiskorbutische Beistoff ist für hohe Temperaturen empfindlich, und zwar so, daß der Grad der Zerstörung abhängig ist erstens von der Dauer, zweitens vom Grad der Erhitzung derselben,

Delf (1918)¹²⁾ hat an Kohlblättern gezeigt, daß ein bedeutender Verlust (80%) an ursprünglich vorhandener antiskorbutischer Wirksamkeit erfolgt, wenn man nur 20 Minuten auf 100° C erhitzt, und daß auch einstündiger Erhitzung auf 100° C, 90% der ursprünglichen Wirk-

samkeit verlorengehen. Die restliche antiskorbutische Substanz ist daher nach 20 Minuten Kochzeit doppelt so groß als nach 1 Stunde. Dr. Delf konnte noch zeigen, daß rasches Kochen bei 100°C dem enthaltenen Antiskorbutikum weniger schadet als langsames Kochen oder Wallenlassen bei $70\text{--}90^{\circ}\text{C}$.

Durch die freundliche Erlaubnis des Herrn Prof. Pirquet und mit Hilfe der Schwester, die der Küche vorsteht, wurde es uns ermöglicht, die Kochart in der Kinderklinik zu studieren.

In Anstalten, wo für eine große Anzahl von Personen gekocht und große Nahrungsmittelmengen verabreicht werden müssen, ist notwendigerweise die Kochdauer eine längere als in kleinen Haushalten. Dazu kommt noch, daß die in Wien übliche Art, Gemüse zuzubereiten, zwei getrennte Kochprozesse zur Folge hat; diese werden nämlich erst weichgekocht und dann vor dem Auftragen in einer sog. „Einbrenn“ aus Mehl und Fett nochmals aufgekocht. Dieses doppelte Kochen bedingt aber eine zweifache Zerstörung des Antiskorbutikums. Auf oben beschriebene Weise wurde nun auch in der Küche der Kinderklinik gekocht. Selbst Kartoffeln wurden häufig zweimal gekocht, erst bei 100°C gesotten oder gedünstet, dann in Scheiben geschnitten und mit Fett und Zwiebeln geröstet. Beim Bereiten der Suppe werden die Rüben, das grüne Gemüse, Tomaten usw. regelmäßig durch 3 bis 4 Stunden einer Temperatur von $90\text{--}100^{\circ}\text{C}$ unterworfen.

Bei dieser Art der Zubereitung der Speisen muß der Verlust an antiskorbutisch wirksamem Material ein sehr weitgehender, wenn nicht vollständiger sein. Es ist daher wahrscheinlich, daß selbst in dem Zeitabschnitt (September 1918 bis Februar 1919), wo ein hinreichendes Quantum an frischem Gemüse beschafft werden konnte, die Kost nichtsdestoweniger dank der durch das Kochen verursachten Zerstörung des Antiskorbutstoffes eine zum Skorbut führende war. Es sind Beispiele in der Literatur vorhanden, in welchen der Ausbruch von Skorbut dem übermäßigen Kochen einer Diät zuzuschreiben ist, welche schon an und für sich arm an frischem Material war (18, S. 65).

Es ließen sich mit Leichtigkeit einige kleine Veränderungen in der Wiener Kochmethode einführen, die, ohne die gegenwärtig übliche Art der Speisenzubereitung wesentlich zu stören, den Verlust an Antiskorbutstoff stark herabmindern würden. Einige Beispiele:

1. Suppen. Jetzt wird bei der Bereitung von Suppe das Gemüse in kleine Stücke geschnitten, ins Wasser getan und das Ganze 3—4 Stunden lang miteinander gekocht. Auch wenn Blattgemüse oder Tomaten verwendet werden, wie bei der „Kohluppe“ oder „Paradeissuppe“, unterliegen diese der ganzen Zeitdauer des Kochens. Man könnte dies in der Weise verbessern, daß man den geschnittenen Kohl

oder die Tomaten usw. erst kurze Zeit, bevor man die Suppe aufträgt, hinzugibt, damit die Erhitzung dieser Gemüse so kurz als möglich dauere.

Wenn frisches Obst oder Gemüse schwer erhältlich ist, könnte man sich helfen, indem man die Rüben auf dem Reibeisen reibt, den Saft auspreßt, die ausgepreßten Rüben mit den übrigen Zutaten zur Suppe so lange als nötig kocht und den rohen Saft erst knapp vor dem Auftragen hinzugibt.

2. Blattgemüse (Kochsalat, Kohl usw.) wird jetzt ausgewaschen, ins Wasser gelegt, zum Sieden gebracht und bei 90–100° C gekocht, bis es weich ist, was ungefähr 1¼ Stunden braucht. Dann wird das Wasser abgegossen und nur ein wenig zurückbehalten, das dem fertigen Gericht beigegeben wird. Das gekochte Gemüse wird gehackt und ein zweites Mal mit einer „Einbrenn“ aus Mehl, Speck, Zwiebeln usw. gekocht und mit Wasser bis zur richtigen Konsistenz versetzt. Es wäre jedenfalls besser, die grünen Blätter nur zu dünsten, statt sie zu sieden. Dadurch könnte die Erhitzung wahrscheinlich auf 20–30 Minuten abgekürzt werden, und der Verlust an Antiskorbutstoff und anderen Vitaminen, die in das nicht zum Genuß gelangende Wasser übergehen, wäre vermieden.

3. Gekeimte Samen. Die Verwendung von gekeimten Hülsenfrüchten und anderen Samen als antiskorbutisch wirksames Zusatzmaterial bei Mangel an frischen Gemüsen kann nicht eindringlich genug empfohlen werden.

Die Zubereitungsart ist folgende: Die Hülsenfrüchte usw. werden mit Wasser ausgewaschen und 24 Stunden lang bei Zimmertemperatur weichen gelassen. Dann legt man sie auf ein gewöhnliches Küchensieb oder eine andere poröse Unterlage, doch dürfen sie nicht höher als 5–7 cm hoch aufgeschichtet werden. Dabei erhält man sie durch gelegentliches Bespritzen mit Wasser unter Luftzutritt 48–72 Stunden feucht. Nach dieser Zeitdauer sollten sich Keime von einem halben bis 1 cm Länge zeigen. Die gekeimten Samen dürfen nicht wieder eintrocknen, sondern müssen gleich gekocht werden. Das Kochen soll nur so kurz dauern, als unbedingt nötig ist, um sie weich und genießbar zu machen. Für kleine Samen, wie Linsen, „Czirok“ usw., genügt es, wenn man sie ½ Stunde einweicht und dann 15 Minuten lang kocht, bei größeren Samen, wie Bohnen und Erbsen, ist wohl eine Kochzeit von 20–30 Minuten erforderlich.

Die hohe antiskorbutische Wertigkeit solchen gekeimten Samenmaterials ist genau durch Tierexperiment (Meerschweinchen) ermittelt worden, und zwar liegt sie, wenn man gleiche Gewichte in Betracht zieht, zwischen der von Kohlblättern einerseits und von Kartoffeln andererseits (Chick und Delf, 1919)^{5, 4)}. Auch bei der

Verhütung und Heilung des menschlichen Skorbutis ist sie schon aufgezeigt worden [Wiltshire 1918²⁰), Dyke 1918¹³]).

Die Tatsache ist beachtenswert, daß trockene Hülsenfrüchte und Czirok während des ganzen von uns untersuchten Zeitabschnittes einen wichtigen Bestandteil der Kost an der Tuberkuloseabteilung bildeten. Während der letzten 3 Monate vor dem Krankheitsausbruch wurde von jedem Kinde täglich im Durchschnitt das Quantum von 33,5 g davon verzehrt (s. Tab. III). Während der Zeit der größten Knappheit, nämlich der 8 dem Auftreten der Krankheit unmittelbar vorausgehenden Wochen, war die tägliche Durchschnittsmenge 30 g pro Kopf. In trockenem Zustande besitzen diese Hülsenfrüchte eine nicht in Betracht kommende antiskorbutische Wirksamkeit, wären sie aber in gekeimtem Zustande verabreicht worden, so hätte die Gabe von 30 g einem Quantum von ca. 60 g frischen Gemüses entsprochen. Diese Menge hätte von unschätzbarem Wert zur Verhütung der Krankheit sein können, selbstverständlich wenn man sie nicht wieder durch zu vieles Kochen unwirksam gemacht hätte.

Tabelle III.

Die in den letzten 3 Monaten vor Ausbruch des Skorbutis und im April 1919 verwendete Menge von Hülsenfrüchten und „Czirok

Monat	Bohnen kg	Erbsen kg	Czirok kg	Summe		Durchschnittsmenge* pro Kopf in gek. Zusta
				trocken kg	gekeimt*) kg	
Januar 1919	55	18,8	21,3	95,1	190	99
Februar 1919	14,8	9,5	40,1	64,4	120	65
März 1919	35,8	2,3	22,4	60,5	121	61
April 1919	62,4	0	0	62,4	125	64

Zusammenfassung.

1. Im April 1919 erkrankten von 64 in der „Sonnenstation“ der Universitätskinderklinik wegen Tuberkulose in Behandlung stehenden Kindern im Alter von 6—14 Jahren 40 an explosiv auftretendem Skorbut.

2. Von den erkrankten Kindern hatte sich Gruppe A mindestens 6 Monate, Gruppe B 3—6 Monate, Gruppe C weniger als 3 Monate vor Ausbruch der Krankheit auf der Klinik befunden. Bei den Gruppe A zusammengefaßten Kindern war die Gewichtszunahme eine normale gewesen, die Kinder der Gruppe B und C hingegen hatten nach der Aufnahme ins Spital besonders rasch an Gewicht zugenommen.

3. Vom September 1918 bis Februar 1919 konnte eine reichliche Menge frischer Nahrungsmittel (über 200 g frischen Gemüses pro Kopf

*) Annehmend, daß bei der Keimung das gleiche Quantum an Wasser aufgenommen wird, was aber wahrscheinlich zu niedrig gegriffen ist.

**) Für 65 Kinder berechnet.

im Tag, abgesehen von frischer Milch) beschafft werden. Von Mitte Februar bis Mitte April wurde das Quantum an frischem Gemüse auf 70 g im Tag herabgesetzt; völliger Mangel trat nie ein.

4. Die achtwöchige Zeitdauer der Einschränkung, die dem Krankheitsausbruch vorausging, ist eine viel kürzere als die von anderen Beobachtern zur Entwicklung von Skorbut als notwendig erkannte, nämlich 4—6 Monate.

5. Bei den Gruppen B und C kann angenommen werden, daß die Entwicklung der Krankheit dadurch beschleunigt wurde, daß der erhöhte Bedarf an antiskorbutischem Vitamin während einer Periode besonders schnellen Wachstums nicht entsprechend befriedigt wurde.

6. Da diese Erklärung für die Kinder der Gruppe A nicht zutrifft, kann man schließen, daß die Zeit des Mangels länger war, als es äußerlich den Anschein hatte, und daß die Kost selbst damals schon eine zum Skorbut führende war, als noch ein genügendes Quantum an frischen Nahrungsmitteln beschafft wurde.

7. Eine Erklärung dafür findet sich in der Art der Speisenzubereitung an der Kinderklinik. Diese hatte eine weitgehende Zerstörung antiskorbutischen Stoffes während des Kochprozesses im Gefolge. Zur Verhütung dieser Tatsache werden einige Vorschläge gemacht.

8. Unter anderem wird dringend empfohlen, die verwendeten Hülsenfrüchte vor dem Kochen zur Keimung zu bringen, als ein Mittel, zu Zeiten, wo frisches Gemüse und Obst selten ist oder ganz fehlt, die Kost an antiskorbutisch wertvollen Stoffen zu bereichern.

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X. DETERMINATION OF THE MINIMUM DOSES OF SOME FRESH CITRUS FRUIT JUICES WHICH WILL PROTECT A GUINEA-PIG FROM SCURVY, TOGETHER WITH SOME OBSERVATIONS ON THE PRESERVATION OF SUCH JUICES.

BY ALICE JANE DAVEY.

From the Department of Experimental Pathology, Lister Institute.

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THE following account deals with work relating to the preservation of fruit juices over long periods, in such a way as to retain the anti-scorbutic principle as little changed as possible.

The work has been carried out under the direction of Dr Harriette Chick, to whom and to Miss Hume I am indebted for much help and advice during the progress of the experiments.

Many experiments by other workers at the Lister Institute have also been included. Some of them have been published already in a summarised form [Chick, Hume and Skelton, 1918, 1, 2], for others I am indebted to my various colleagues at the Lister Institute.

INTRODUCTION.

Early in 1917, in the course of experiments concerned with the relative anti-scorbutic values of fruit and vegetables, particularly with a view to the needs of the Army, official samples of preserved lime juice, as supplied to the Army and Navy, were tested. The results showed that such preserved lime juices were practically devoid of anti-scorbutic principle, since the largest doses (10 cc. daily), that could be administered, did not indicate the slightest protection of guinea-pigs from scurvy, whereas a very much smaller dose (1.5 cc. daily) of fresh orange or lemon juice will suffice to keep a guinea-pig in good health.

It was thought that the failure of the lime juice might be due to deterioration induced by long keeping or by the method of preservation. Accordingly, tests were instituted on samples of crude juice supplied by the manufacturers and on fresh juice expressed in the laboratory from imported fruit. It was found that the fresh lime juice possessed not more than one quarter of the value of fresh lemon juice [see Chick, Hume and Skelton, 1918, 1, 2].

This result raised the question of the generally received belief in the efficacy of lime juice as a preventive of scurvy and investigations were set on foot to determine the relative anti-scorbutic values of the commoner Citrus fruit juices, and the extent to which these values were diminished by the various methods of preservation used and by long keeping at different temperatures. Attempts have been made to find a method of preserving lemon juice which shall reduce this deterioration to a minimum.

There have already been published the results of the enquiry just referred to, by Chick, Hume and Skelton [1918, 2] into the relative content of anti-scorbutic principle in lemons (*Citrus medica* var. *limonum*) and limes (*C. medica* var. *acida*) where the summarised results include experiments with both guinea-pigs and monkeys. Both sets of experiments clearly demonstrate that "the value of fresh lemon juice is approximately four times that of fresh lime juice" and that preservation and long keeping of lime juice induce considerable loss of its anti-scorbutic principle.

The conflict between experimental results on the one hand and human tradition and practice on the other was resolved by the historical enquiry made by Mrs Henderson Smith [1918, 1919], regarding the use of lime and lemon juice in connection with human scurvy.

More recently, the complete failure of lime juice to afford protection to the army in Mesopotamia is a well-known fact [Willcox, 1920] and later still in February and March 1919, Stevenson [1920] made the same observation of its worthlessness, among the inmates of the Russian civil prisons at Archangel.

PREVIOUS EXPERIMENTAL WORK ON CITRUS FRUIT JUICES.

Old records contain many instances of the great value of oranges and lemons for the prevention and cure of scurvy. One of the most striking is the classical human experiment carried out by Dr Lind [1757; quoted in the Medical Research Committee's Report, 1919, p. 39] by which he showed that oranges and lemons were far superior to other remedies, current in his time.

Some experiments have been carried out by Holst and Frölich [1912] on the anti-scorbutic value of lime and lemon juices, using guinea-pigs as experimental animals. These workers compared freshly squeezed raw lemon juice with commercial samples of lime juice preserved in various ways. The animals were fed on a basal diet of grain and water only; the dose of fruit juice was 5 to 10 cc. daily at most. Comparison was made with other animals receiving no anti-scorbutic. Very little protection was afforded from scurvy and the juice of fresh lemons was found little if at all superior to the preserved lime juices. The addition of the juices to the diet of grain and water prolonged life and mitigated the scurvy symptoms slightly, hence anti-scorbutic in small amount was assumed to be present in the doses given. Holst and Frölich regard this result as lending additional support to the view that guinea-pigs are more susceptible than man to scurvy, and point out that a daily dose of 5 to 10 cc. of lime juice for a guinea-pig, if related to the body weight, is a

proportionally much greater dose than that held to be sufficient to protect a man from scurvy. Since the slight protective effects of the preserved lime juice were not found to be markedly inferior to those of the fresh lemon, the authors inferred that lime juice retains most of its potency after preservation and keeping, and they assume that their experiments justify the widespread belief in the usefulness of preserved lime juice, which they never seem to have called in question.

On the basal diet of grain and water used by Holst and Frölich, with addition of anti-scorbutic in the form of lemon juice, the animals grew very little and hunger symptoms were apparent, since the animals failed to consume the grain. Some of the earliest experiments of Chick and co-workers (Chick and Hume, 1917), showed that if guinea-pigs received a basal diet of oats and bran and water with anti-scorbutic in the form of fruit juice, good health and growth could not be maintained. The animals failed to consume sufficient food and often could not be induced to take their ration of lime or lemon juice without ill effect. The diet cannot be considered equivalent to one in which a ration of fresh cabbage (containing all the accessory food factors) takes the place of the fresh fruit juices. Hence no conclusion drawn from such experiments as to the relative anti-scorbutic potency of, for instance, cabbage and lemon juice can be regarded as valid.

SCOPE OF THE PRESENT PAPER.

The present paper deals first with the determination of the minimum doses of fresh fruit juices, orange, lemon and lime, required to prevent scurvy in guinea-pigs. The results have been used as a basis for finding the loss in anti-scorbutic value which takes place when these juices are preserved or kept for long periods at different temperatures.

Besides an investigation of the keeping properties of carefully preserved laboratory material, the results are also included of tests on several commercial and official samples. Since the true history and mode of preparation of these is not always known the results lack scientific value, but are of very great importance from the practical standpoint.

Some data are also included for the value of chilled fruit. The whole investigation is still not quite complete since few of the specially prepared specimens have been kept as yet for more than two years. Such a period of preservation is however sufficient to give some good indications of the best practical methods to adopt.

TECHNIQUE.

In all particulars the method employed is that described by Chick, Hume and Skelton [1918, 2] and also by Delf and Tozer [1918]. All the experiments have been carried out on young growing guinea-pigs (about 340 g. in weight at the beginning of the experiment) fed on a basal diet of oats and bran, to which was added a ration of 60–90 cc. of cow's milk, autoclaved at 120° for an hour. The fruit juices tested were given as daily doses, administered

by pipette. All food residues were weighed or measured so that the quantities consumed daily could be recorded. The animals were weighed three times weekly. Symptoms of scurvy were noted during life and at post mortem.

The symptoms of guinea-pig scurvy are fully described by Chick, Hume and Skelton [1918, 2] and by Delf and Tozer [1918]; see also the Report of the Medical Research Committee on Accessory Food Factors [1919].

In the present paper the presence or absence of haemorrhages is taken as the decisive symptom in diagnosing scurvy or the reverse. A condition of histological bone lesion, in absence of haemorrhages, is not taken as indicative of scurvy in the light of the results obtained by Tozer [Delf and Tozer, 1918], which show that such a bone lesion frequently also occurs in animals on non-scorbutic diets and is probably due to a deficiency of vitamin A.

In order to avoid the ill effects on the animals of large doses of acid juices, all lime juices and certain of the more acid preserved lemon juices were partly, never wholly, neutralised with solid sodium carbonate. Lime juice was rendered more palatable by the addition of cane sugar (1 g. to 10 cc. of juice). Lemon or lime juice doses, whether neutralised or not, were mixed with a little auto-claved milk from the animal's ration before administration. Neutralisation was carried out as short a time as possible before administering doses to the animals, and it is assumed that no appreciable change in the anti-scorbutic value of the ration was so induced [see Harden and Zilva, 1918].

Juices used for experiment were always kept in the Lister Institute refrigerator during experiment in order to keep them as uniform as possible throughout the experiment. The temperature of the refrigerator usually ranged from -3° to 4° . On rare occasions it went down to -5° and once rose for a week to 18° .

DETAILS OF EXPERIMENTS AND RESULTS.

I. DETERMINATION OF MINIMUM DOSES OF FRESH JUICES.

A. Lemon Juice (*Citrus medica* var. *limonum*).

Sound ripe fruit was selected, any soft or blemished specimens being rejected. The large rough skinned varieties were used and efforts were made to secure uniformity as far as possible. The fruits were halved and the juice expressed by hand on a glass squeezer and strained through coarse muslin or mosquito net with firm squeezing. Juice thus prepared was kept in the refrigerator, but not frozen, during the period of testing. For the earlier experiments [Chick, Hume and Skelton, 1918, 1] supplies were made monthly, so as to be comparable with lime juice from imported fruit, which could not be obtained more frequently. In order to eliminate possible changes due to keeping even for a short time, the juice for the present experiments was prepared fresh every week.

Experiments have been carried out in order to determine within narrow limits, the minimal daily protective dose for a guinea-pig. Early unpublished

experiments, conducted in 1917, show that 10 cc. and 5 cc. daily doses afford ample protection. The paper published in 1918 recorded the effects of 2.5 cc., 1.5 cc. and 0.5 cc. daily doses and 1.5 cc. was found to be the minimal protective dose. The present experiments, using fresher juice, also test the same three doses and reach the same conclusion.

2.5 cc. daily dose. Six animals were employed. Two of these died from illnesses other than scurvy after 33 and 37 days respectively. The remaining four animals completed the experimental time and were killed in good health. They were all four in splendid condition, but two of them which had consumed a larger milk ration (85 and 95 cc. respectively, during the last 60 days of experiment), continued to increase in weight to the end of the experimental time and attained a higher maximum weight than did the others.

1.5 cc. daily dose. Six animals were used. Of these one died after 30 days of an acute intestinal affection; no scurvy was indicated. The others completed the experimental time (90 days) in good health. The post mortem indicated protection from scurvy.

0.5 cc. daily dose. Six animals were used. All developed scurvy. Two of them were killed after 45 days. Attempts were made to cure three of the others with the juice of sweet limes (see p. 90), while the remaining one was treated with canned apricot juice.

The 0.5 cc. daily dose is manifestly insufficient, but some slight protection is indicated because symptoms in life were not diagnosed earlier than the 21st to 28th day, and the length of life in cases where no cure was attempted, was not less than 40 days.

It is concluded therefore that a daily ration of 1.5 cc. fresh lemon juice (never more than one week old) is the minimum daily dose which will protect a guinea-pig from scurvy and this datum is used for the basis for comparison with the anti-scorbutic value of other fruit juices or of different preserved lemon juices. The result of this experiment is very slightly better than that detailed by Chick, Hume and Skelton [1918, 1] where the juice used was kept 0-2 months in the refrigerator, a result which suggests an appreciable though slight loss of anti-scorbutic principle when the juice is kept for such a period even at a low temperature.

B. Lemon Juice with the Rind Oil.

Experiments on preservation which are detailed later had shown that lemon and orange juice, squeezed so as to contain some of the essential oil of the rind, would keep sweet and palatable for long periods, without addition of other preservative, in the same way as lime juice, so treated, does. Lemon and orange juices squeezed without such precaution and without any other preservative, quickly become mouldy and unpalatable. Experiments to determine the minimum dose of such material when fresh are therefore included here with the determination of the minimum doses of the other fresh fruit juices.

The material was prepared in the following way. Whole lemons were sliced by hand and minced in an ordinary kitchen mincer. The juicy pulp thus obtained was squeezed by hand through coarse muslin. The resulting "juice" is a thick creamy emulsion which sets to a soft curd, from which a small amount of clear juice slowly separates. The curd is easily broken up later. This material was bottled without any sterilising precautions and seems to keep well even when the bottles are frequently opened.

As compared with fresh lemon juice expressed without the rind oil, this material is diluted somewhat with the rind constituents, *i.e.* the solid matter of the white inner rind and its watery sap.

The material was kept in the refrigerator (about 0°) during the period of the experiment, *i.e.* about 100 days.

For convenience in measuring small doses of the very thick material, the total quantity required each day was diluted with an equal volume of water; twice the volume of the prescribed dose was then administered to each animal.

1.5 cc. daily dose. Four animals were used; one of these showed severe scurvy after 34 days, while another completed 90 days of experiment but showed some indications of scurvy of long standing. Two others were completely protected for 90 days. 1.5 cc. of this juice was not, therefore, quite adequate.

2.5 cc. daily dose. Four animals were used of which two died early from intestinal troubles (32 and 34 days). The two remaining animals completed the experiment in good health, without signs of scurvy at post mortem.

The minimum daily dose of this lemon juice with the rind oil (0–10 days old) which will protect a guinea-pig from scurvy is therefore between 1.5 and 2.5 cc.

C. Orange Juice.

The juice was prepared exactly as is described for fresh lemon juice. Some difficulty was experienced in obtaining uniformity owing to the variation in quality of the oranges according to the season of the year. This accounts for certain discrepancies in the results. Oranges deteriorate more rapidly than lemons and late in the season they become dry. The critical experiments on which minimum dose values have been based, were carried out early in the year (Jan. to March), using as far as possible oranges of the same variety (Denia). The juice was prepared once a week, in certain experiments twice a week, and was kept in the refrigerator between successive times of preparation.

Experiments conducted in 1917, using daily rations of 10 cc., 5 cc. and 3 cc., show that these are amply sufficient to protect a guinea-pig from scurvy. Some of these experiments are mentioned in an earlier paper from the Lister Institute [Chick and Hume, 1917]. Further experiments have been conducted and are detailed below, using 1.5 cc. and 0.5 cc. daily doses.

1.5 cc. *daily dose*. Three series of experiments have been conducted at different seasons of the year.

(a) Experiment extending from May to July; three animals were used; the juice was expressed weekly. Of the three animals, one died from scurvy in 50 days; another showed "doubtful scurvy" without haemorrhages after 83 days, while the third succumbed to an intestinal illness after 63 days, but was completely protected from scurvy.

(b) Experiment extending from March to May; four animals were used; the juice was expressed weekly. Only one animal survived 90 days. It was in excellent health and was completely protected from scurvy. All the other cases were complicated by intestinal infections. No scurvy symptoms were observed during life or at the post mortem, but as the animals died or were killed after 46, 62 and 67 days, the result is not conclusive.

(c) Experiment extending from January to March; four animals were used; the juice was expressed twice weekly. One animal died of pneumonia after 67 days but some scurvy was found at post mortem. Two others died from intestinal disorders after 74 and 88 days respectively. At the post mortem one of these showed fragility of bones but no haemorrhages, while the other was without any signs of scurvy. The remaining animal survived 90 days and at the post mortem was found to be normal.

The above results may not appear entirely conclusive; out of a total of eleven animals six were protected from scurvy and of these four survived more than 80 days of experiment. The remaining two, as well as the other partially protected animals, were all victims of infections or illnesses other than scurvy. Experiment (c), in which oranges were at their best and the juice used never more than four days old, gives a substantially better result than the others; only in one case was there any scurvy indicated at the post mortem, and that by fragility of the bones only, haemorrhages being absent. In fact three out of the four animals were protected.

0.5 cc. *daily dose*. An experiment with one animal was begun in June 1918. Oranges were then dry and their anti-scorbutic potency lowered as shown by the experiments described above with larger doses. The animal died on the 58th day of experiment with moderately severe scurvy complicated by other illness.

A further experiment was begun in March 1919, the juice being prepared twice weekly. Five animals were employed, all of which showed signs of scurvy round about the 34th day of experiment. One of them showed scurvy with severe haemorrhages and fragile bones after 57 days; another died from disease other than scurvy after 43 days; scurvy haemorrhages were not found but the bones were slightly fragile and the rib junctions nodular. The remaining animals were suffering from severe scurvy from the 57th to the 67th days, as indicated by sore swollen joints, lameness etc. At this time their doses were changed to a daily ration of sweet lime juice which effected a partial cure.

From these experiments it is assumed that 1.5 cc. daily may be considered a minimal protective dose for a guinea-pig, when the freshly expressed juice of oranges at their best, *i.e.* in the early part of the season, is used.

D. Orange Juice with the Rind Oil.

Experiments with lemon juice with the rind oil were so promising that material was prepared in exactly the same way from oranges, in order to establish the minimum dose and test the keeping capacity of this material also.

The fruit was obtained early in March 1919; it was treated exactly as is described for lemons. The emulsion was very thick and set to a substantial curd, a small proportion of juice squeezed without the rind oil was therefore added.

The material was kept in the refrigerator at a temperature about 0° for the time of the experiment, *i.e.* 0-108 days.

1.5 cc. daily dose. Four animals were used, of which two were in good health at the end of the experiment. Two died from other causes (63 and 72 days) with very doubtful scurvy, slight bone brittleness but no haemorrhages.

3 cc. daily dose. Two animals were used and both were normal at the end of the experiment. The minimum dose of orange juice with the rind oil kept in the refrigerator for 0-108 days was therefore taken to be 1.5 cc. daily.

E. Sweet Lime (*Citrus medica* var. *limetta*).

A small sample of sweet limes was received from Basra in February 1919, sent by Lt.-Col. Ledingham. The sample arrived in poor condition owing to the long time taken in transit. Many of the fruits were useless and the remainder though placed in the refrigerator, deteriorated rapidly and became mouldy and soft. Juice was expressed every two or three days.

It was tested as a cure for animals sick with scurvy, induced by insufficient doses of fresh lemon and orange juices.

Of the six animals used, three had been on a daily ration of 0.5 cc. fresh lemon juice for 49, 52 and 48 days respectively, while three had been on a similar ration of fresh orange juice for 57, 65 and 67 days respectively. All were suffering from severe scurvy as evinced by swollen, painful joints, scurvy position, lameness and falling weight. A daily ration of 2.5 cc. of the juice of sweet limes was substituted for the orange and lemon doses. A very gradual improvement in the condition of the animals became apparent with steadying of the previously falling weight curves; in some cases slight gain in weight took place. Although the cure was very far from complete, the life of the animals was very considerably prolonged. The average life of the six animals in question was 78 days and two of them survived 89 and 91 days respectively; whereas the average length of life of five animals which continued on the small doses of orange and lemon was 49 days only. The post mortem

examination revealed some repair of bone lesions and absence of recent haemorrhages, although signs of severe old haemorrhages were apparent.

Although the sweet limes were old and in very poor condition their juice exercised considerable curative power when administered in 2.5 cc. daily doses to guinea-pigs suffering from severe scurvy. No definite conclusions can be drawn as to the value of sweet limes in good condition, but the result obtained suggests that they should be useful as an anti-scorbutic and superior in this respect to sour limes.

F. Sour or West Indian Lime Juice (*Citrus medica* var. *acida*).

The experiments with fresh ripe lime juice were conducted during the winter of 1917-18 and have been summarised by Chick, Hume and Skelton [1918, 1, 2]. The results are included again here, with some further notes for comparison with the newer results from preserved lime juice and juice from green limes.

The fruit used was obtained through the kindness of Messrs L. Rose & Co. who furnished monthly supplies of specially imported West Indian limes. Owing to war conditions these limes were from one to two months in transit. They do not seem to bear keeping as well as lemons, possibly because of their thinner rind, from which the volatile essential oil is more easily lost.

The juice was prepared exactly as described for lemon and orange juices without the rind oil, only sound firm fruit being employed in its preparation.

The experiment included tests with green unripe limes as well as with ripe limes, as it was thought that the anti-scorbutic value of the green fruit might be greater than that of the ripe fruit. We can draw no very definite line as to what constitutes the limit of ripe and unripe fruit, but ripe fruits were definitely yellow.

Experiments were conducted with 2.5 cc., 5 cc. and 10 cc. daily doses of fresh lime juice. The doses were half neutralised with sodium carbonate, and cane sugar (1 g. per 10 cc. juice) was added to render the juice palatable.

The age of the juice after squeezing varied from 0 to 30 or 40 days old, it being preserved for that time in the refrigerator between squeezings.

2.5 cc. daily dose. (a) Ripe lime. This amount of ripe lime juice afforded no protection from scurvy. Of seven animals, five died from severe scurvy in 23 to 59 days. The remaining two cases were complicated by other illness and scurvy was less severe after 29 to 67 days, than in the preceding cases.

(b) Green lime. When the juice from green limes was employed the results were appreciably better. Scurvy symptoms were milder and the duration of life was longer. One of the four animals used lived for 116 days suffering from slight chronic scurvy, with some improvement towards the end of the experiment, probably correlated with the use of a fresh sample of juice. In another animal which died from infection after 38 days, the post mortem showed no sign of scurvy. The two remaining animals showed scurvy of moderate severity after 56 and 85 days respectively.

5 cc. *daily dose.* (a) Ripe lime. Ten animals were used of which three showed definite scurvy and three were normal; two showed doubtful scurvy and two died from other causes (26 and 35 days) without haemorrhages.

(b) Green lime. Seven animals were used, of which three died within 34 days from other illness than scurvy and are therefore discounted. As with the ripe lime, practically complete protection was obtained in three of the four cases remaining; the animals survived for over 90 days and no haemorrhages were found at post mortem. The fourth animal showed only doubtful traces of scurvy after 56 days.

10 cc. *daily dose.* Ripe lime only. Doubling of the 5 cc. dose produced no appreciably better result and it failed to eliminate the slight fragility of the bones and enlargement of the rib junctions, with the corresponding histological symptoms, which so frequently occurred but which there is little doubt were due to an insufficiency of vitamin A.

Of six animals one was normal in all respects after 88 days of experiment. Another died with severe visceral haemorrhage after 85 days, showing fragility of bones at post mortem. Scurvy was doubtful or absent (no haemorrhages) in two other cases after 52 and 62 days respectively and two died without haemorrhages at 25 and 34 days from other causes.

It must be noted that animals receiving lime juice never grew as vigorously as the animals receiving orange juice or the smaller doses of lemon juice. Healthy animals usually attain a maximum weight of from 550 to 600 g. within the 90 days of experiment, their weight curve approximating nearly to that of animals fed on normal diet. With one exception the highest maximum weight reached by any animal on a 5 cc. or 10 cc. fresh lime juice ration was 470 g. The exception was the single completely protected animal on the 5 cc. daily ration of fresh ripe lime juice, whose weight reached 537 g. on the 78th day of experiment.

The experiments with fresh lime juice indicate 5 cc. daily as the minimal protective dose for a guinea-pig. Taking into consideration the poor growth and consequent low body weight of even those animals which were completely protected from scurvy, it is possible that the 5 cc. daily dose might not ensure protection to better grown animals, whose weight curve more nearly approximated to the normal.

On the other hand, conditions necessitated the use of lime juice prepared monthly from fruits which had suffered from delay in transit. If tests be made from lime juice prepared at more frequent intervals from fresh fruit, the results may indicate a slightly lower value for the minimal dose than has so far been obtained. Pending such further experiments, the minimal protective dose of fresh lime juice required by a guinea-pig cannot be fixed at less than 5 cc. daily, which amount is perhaps best regarded as marginal.

II. PRESERVATION OF FRUIT JUICES.

A. Official and Commercial Samples.

The work carried out with official or commercial samples chiefly concerns the juices of lemon and lime, on account of their use in the rationing of armies, arctic expeditions etc. and the consequent urgent need during the late war for exact information as to their anti-scorbutic value.

Tests have been carried out with a number of official samples as supplied to the Army and Navy, obtained through the authorities. Various commercial samples preserved by different methods have also been tested. Details with regard to a number of such samples have already been published [Chick, Hume and Skelton, 1918, 1]; a few more tests of special samples are added here and the tests of a certain number of similar samples of lemon juice.

Acknowledgments are here made to Messrs L. Rose & Co. and to Messrs Evans Sons, Lescher & Webb for their kindness in supplying samples and furnishing data as to age and method of preparation.

In view of the proved inferiority of lime juice to lemon juice in the fresh state and the uselessness of the preserved lime juices, and having regard also to the promising results obtained with preserved lemon juice, it was recommended to the authorities that steps might be taken to substitute lemon juice for lime juice in supplies to the Army and Navy, thus reverting to the usage of the early part of last century. In response to this suggestion, samples of lemon juices submitted to the authorities have been tested on their behalf.

Since there is some evidence that greater care in the method of preservation will allow retention of more of the anti-scorbutic potency, careful comparison has also been made of the above-mentioned lemon juice with lime juice specially prepared by Messrs Rose, on exactly the same lines as were recommended and adopted in preparing the samples of lemon juice.

A 1. Official and Commercial Samples of Lime Juice.

Reference to the table given by Chick, Hume and Skelton [1918, 1, Table I] shows that of six samples of crude lime juice examined, none showed any protection in 5 cc. daily doses, save two samples (Nos. 3 and 4 in table) which did show some small degree of protection. In the case of two of the samples however, even a 10 cc. daily dose conferred no protection.

Two further samples, Nos. 7 and 8, were examined in the course of the present work and yielded a similar result. These were both crude juice supplied by Messrs Rose; the one (No. 7) was prepared from ripe limes in the usual way save that none of the essential oil of the rind was removed before crushing and squeezing; the whole fruit was squeezed right out and the juice, containing more than the usual amount of essential oil, was run straight into the casks for export; two animals on 5 cc. and two on 10 cc. of this material all showed scurvy.

The second sample (No. 8) was ordinary crude lime juice containing no

more than the normal amount of essential oil but prepared from green limes instead of ripe ones. Four animals received 5 cc. of it daily and all showed scurvy. In neither of these cases was the juice more than 2-3 months old when the experiment started.

A third sample (No. 9) was prepared with especial care by Messrs Rose so as to be comparable with the special sample of lemon juice submitted on behalf of the War Office. The material appeared to be crude juice containing the rind oil and 0.07 % SO_2 , added as preservative. This sample of lime juice gave the best result ever obtained with lime juice. The lowest value hitherto obtained for the minimum daily dose of lime juice needed to protect a guinea-pig from scurvy is 5 cc., even with juice expressed from imported, though not very fresh limes. With this dose not all guinea-pigs were protected. The special sample, at present under consideration, gave a value rather less than 5 cc., all animals on 5 cc. being perfectly protected; animals on 2.5 cc. all showed scurvy. The inferiority of lime juice is thus still upheld, though in this special case it is considerably less marked than in any other sample ever tested.

A 2. Official and Commercial Samples of Lemon Juice.

Four different samples were examined and of these three gave good results, showing a far higher degree of protection on a 5 cc. dose than did the commercial samples of lime juice.

No. 10 was a sample of lemon juice prepared by Messrs Rose for the War Office, squeezed with the rind oil and with $\text{SO}_2 = 0.1\%$ as preservative. This sample corresponds with the lime juice sample No. 9. Protection was obtained in two cases, and a doubtful result in the third case with a 1.5 cc. daily dose of this material, a result practically equal to that obtained with the freshly expressed juice.

No. 11. Lemon juice, expressed without the rind oil, preserved in Messina with 0.25 gal. sulphurous acid to 100 gals. of juice. This juice had been kept for at least six months at room temperature. Only a 5 cc. daily dose was administered and this gave undoubted protection in three cases out of four, the fourth being uncertain. Considerable loss may therefore have taken place but it cannot have been by any means complete.

No. 12. A lemon juice sample furnished by Messrs Evans Sons, Lescher & Webb. It was received in October from lemons squeezed in the preceding spring in Sicily. No preservative was added and apparently none of the rind oil. Only 5 cc. and 10 cc. daily doses were administered and in each case no scurvy developed. Loss, if it had taken place, was therefore not great.

No. 13. A sample of "Kia-Ora" lemon squash, bought in a shop. The history was unknown. Sugar had been added. Doses of 5 and 10 cc. showed no protection from scurvy. This sample therefore ranks with the commercial samples of lime juice, all the other lemon juices being very much superior.

It is quite clear from the results so far given that lemon juice is much

more potent anti-scorbutically than lime juice. It is not however possible to make an accurate quantitative comparison of the keeping capacities of the two, for it is easy to give to guinea pigs a 5 cc. dose of lemon juice, which gives the information whether lemon juice has lost two-thirds of its anti-scorbutic value or not, but it is impracticable to give 15 cc. of lime juice, which is the daily dose necessary in order to obtain the same information about lime juice.

B. Preservation of Special Samples.

The foregoing experiments show clearly (a) that orange and lemon juice are more potent as anti-scorbutics than lime juice, and (b) that they are on this account more suitable material for use in keeping experiments where gradual loss of anti-scorbutic potency is to be anticipated.

Samples of lemon juice prepared from fresh fruit were stored in the Lister Institute refrigerator, without addition of any preservative. They were tested after an interval of seven months, and it was found that a daily dose of 5 cc. conferred almost complete protection on a guinea-pig. Such juice retained its value even after fermentation of the sugar had taken place, a 5 cc. daily dose again conferring considerable protection. This result stimulated further the enquiry into the methods of preserving and keeping lemon juice, and experiments have been carried out with juice prepared in the laboratory, while others are still in progress. As it was believed that the temperature at which the juice was stored would make considerable difference to the anti-scorbutic, as well as to the gross keeping capacity of the juice, samples were stored at different temperatures. Experiments with samples of lemon juice preserved with varying percentages of sulphite and with the rind oil and of orange juice with the rind oil are also given.

B 1. *Preservation of Lemon Juice by means of "Sulphite"* (potassium metabisulphite).

The juice was expressed from carefully selected fruit, exactly in the manner described for fresh fruit juices. Quantities of from 20 to 30 litres were prepared at one time and the process occupied one or two days. The sulphite was added in 10 % aqueous solution, the juice being stirred vigorously to ensure complete mixing. The juice was bottled immediately, stoppers or corks being sealed with wax so as to be airtight. Two strengths of preservative were tried giving final concentrations of 0.06 % and 0.09 % sulphite in the juice. In the case of a sample of juice to be kept at a higher temperature 0.1 % sulphite was used. It has been found that juice preserved with sulphite does not keep well after bottles have once been opened, hence it is best to store it in bottles containing quantities suitable for a single experiment. Sterilising of bottles previous to filling made no appreciable difference and was accordingly dispensed with.

Samples of this material have been stored for periods of many months, under the following conditions:

(a) At ordinary room temperature (12° to 25°), in a dark cupboard in a corridor, not subjected to sunlight.

(b) In a hot room at 37° .

(c) In the Lister Institute refrigerator, where the temperature was usually slightly above 0° but ranged from -3° to 4° , once falling as low as -5° and once (owing to a breakdown) for a week rising to 18° . In winter the juice was occasionally frozen.

The preserved juice is a clear pale yellow liquid containing a small amount of curdy sediment. When efficiently sealed the material does not darken perceptibly after 12 months' keeping in the cold room or at room temperature, but in the hot room considerable darkening takes place in the course of a few weeks although the juice continues for some time to remain good as far as taste and smell are concerned; ultimately however it becomes bad and quite unpalatable. The samples to be tested were removed from the place of storage at the beginning of the experiment and kept in the refrigerator while the experiment progressed.

Tests have been carried out as shown below.

(a) Lemon juice preserved with sulphite, kept at room temperature.

1. *Material preserved with 0.06 % sulphite.*

Two batches of this material were made. The first batch (No. 14) had been entirely used up after eight months; a second batch (No. 15) was therefore made for further keeping.

Age $3\frac{1}{2}$ to 5 months (No. 14). After $3\frac{1}{2}$ to 4 months, a 2.5 cc. daily dose failed to protect. After 4 to 5 months a 5 cc. daily dose failed to protect, but the juice appeared to have gone bad, probably due to faulty sealing and all the four animals used developed scurvy as though no anti-scorbutic had been given. On the 19th and 25th days respectively, two of them had their doses replaced by 5 cc. of the juice left over from the last experiment. This seemed to check falling weight in one case but after 14 days both the animals showed advanced scurvy, being thin and miserable. Their dose was then changed to 5 cc. of juice from an unopened bottle of the same material. After 14 days the condition of both animals improved remarkably; there was rise of weight and the use of the hind limbs was regained; they were then killed and showed good condition and repair of bone lesions.

Thus a 5 cc. daily dose, after four months' keeping at room temperature, is in some cases sufficient to effect a cure in cases of advanced scurvy. This indicates a deterioration to about one-third of the original value.

Age 6 to 10 months (No. 14). A 5 cc. daily dose gave almost complete protection. Of the four animals used, one died from disease other than scurvy in 30 days. Three animals completed the experiment (88-90 days). There

was evidence of slight scurvy, some fragility of bones and slight haemorrhage being found at post mortem.

A further test was carried out using 10 cc. daily. Only one animal was employed. It never made very good growth, remained thin and in poor condition and died after 61 days. Slight fragility of bones was found, but the animal was protected from scurvy.

None of the above-mentioned animals received more than 60 cc. of auto-claved milk daily. Animals receiving large daily rations of the juice preserved with sulphite never made good growth and failed to reach the weights attained by normal animals or by those on small rations of fresh juice. In subsequent experiments the animals received a larger milk ration and the doses of lemon juice were half neutralised with sodium carbonate.

Age 18 to 22 months (No. 15). Animals receiving a 5 cc. and a 2.5 cc. daily dose showed good health after 60 days; the experiment was unfinished at the time of going to press but it seemed likely that the 2.5 cc. daily dose would afford protection, showing that in this case after nearly two years' keeping at room temperature, lemon juice preserved with 0.06 % sulphite, in some cases at any rate, retains at least three-fifths of its anti-scorbutic potency (the average minimum protective dose of fresh lemon being 1.5 cc.).

2. *Material preserved with 0.09 % sulphite.*

Age 7 to 12 months (No. 16). At seven months a 5 cc. dose effected protection. At 12 months a 2.5 cc. dose failed to do so.

Age 19 to 21 months (No. 16). At 19 months a 5 cc. dose failed to protect. At 21 months two animals on a 10 cc. dose were in good health after 50 days of experiment; the experiment was still unfinished at the time of going to press.

Material preserved nearly two years with 0.09 % sulphite at room temperature therefore, in this particular set of experiments, showed signs of slow, steady deterioration down to one-fifth or one-sixth of its original value (taking the minimal dose of fresh lemon juice as 1.5 cc.). The various results with lemon juice preserved with sulphite are however rather inconsistent and the different points are more fully discussed later.

(b) *Lemon juice preserved with sulphite, kept at 37°.*

This material was prepared in April 1918 and contained 0.1 % of sulphite. Tests were carried out after three months' keeping, when the juice was already very dark in colour and after 28 months.

Age 3 months (No. 17). Four animals received 5 cc. daily. One of these died from infection after 30 days but it is doubtful whether scurvy was beginning. Another animal showed complete protection from scurvy after 67 days. The remaining two survived for 89 days, one with slight scurvy and the other with severe scurvy, the onset of which dated from about the 67th day.

Although insufficient for complete protection the 5 cc. dose was of considerable value.

Age 28 months (No. 17). There was no evidence of any protection when a daily dose of 10 cc. was given, the two animals used dying of scurvy in 23 to 30 days. Thus the material when kept at a temperature of 37° showed no anti-scorbutic value after two years; it was also bad in the gross sense.

(c) Lemon juice preserved with sulphite, kept in the refrigerator (about 0°).

This material contained 0.06 % of sulphite and was the same as that used as No. 14. Tests were carried out on it after 5 and 27 months' keeping.

Age 5 months. A 5 cc. daily dose afforded complete protection to four animals.

Age 27 months. A 2.5 cc. daily dose afforded complete protection to two animals. Of two animals receiving 5 cc. one was completely protected and one which became ill from other causes showed slight symptoms of scurvy; doubtless this anomaly, in which the 2.5 cc. dose appears more effective than the 5 cc. was due to the interference of intercurrent disease. In the case of this material stored for more than two years at about 0° , the anti-scorbutic loss seems very slight.

Summary of results, obtained with lemon juice preserved with sulphite.

On reference to the details (Nos. 14, 15, 16) of material stored at room temperature, it is seen that no daily ration of less than 5 cc. was capable of giving adequate protection to a guinea-pig from scurvy, except possibly in the case of the unfinished experiment with 2.5 cc., 22 months old (No. 15). After the juice had been kept only four months, the 5 cc. dose was barely adequate while half this amount was insufficient. Nevertheless, after 18 months' keeping, a 5 cc. daily dose of lemon juice containing 0.06 % of sulphite afforded complete protection.

The effect of the time factor during periods of less than three months has not been investigated. There is considerable loss of anti-scorbutic within this period, since the minimum daily dose of fresh lemon juice is 1.5 cc. daily.

There is no striking difference corresponding with the different percentages of sulphite employed, but the larger amount is probably to be recommended.

The results contain one striking discrepancy, namely that while 5 cc. of lemon juice, containing 0.06 % sulphite, constituted a protective dose after 18 months' keeping, the same dose of similar material, containing 0.09 % sulphite, was powerless to prevent scurvy. Such inconsistencies as this, considered together with the ready tendency of sulphite material to deteriorate after exposure to air, suggest that the sulphite method of preservation is unreliable, in some cases giving good results but in others failing completely.

The keeping experiments at higher temperatures were carried out in order to test the possibility of preserving lemon juice for use in hot climates. As

might be expected, the destruction of anti-scorbutic goes on more rapidly than at lower temperatures. Nevertheless the material was not entirely useless after three months' storage, when a daily dose of 5 cc. afforded considerable protection from scurvy. After 27 months' storage however, no value at all could be detected.

Preservation at lower temperatures increases the time for which the juice will remain efficacious. Extreme limits have not yet been determined. After 27 months' keeping, a 2.5 cc. daily dose of material stored in the cold room protected a guinea-pig from scurvy.

B 2. *Preservation by means of the Rind Oil.*

Experience with lime juice showed that when the juice is expressed from the whole fruit so as to contain the essential oil of the rind it keeps well in the gross sense, without the addition of foreign preservatives. It seemed probable that this method might also serve for other Citrus fruit juices. Small samples of such juice expressed from minced lemons were found to keep well in the laboratory so far as taste and smell are concerned. It remained to test how far the anti-scorbutic value was at the same time preserved.

Accordingly large quantities of material were prepared for testing in the way already described under the determination of the minimum dose for orange and lemon juice with the rind oil. Arrangements were made to carry out tests at considerable periods on material stored at room temperature, at 37° and at a temperature round about 0° (in the Lister Institute refrigerator) in the case of lemon juice, and at 37° and room temperature for orange juice. Material from whatever source was, as usual, stored in the refrigerator during the actual course of each individual test.

(a) Lemon juice with the rind oil.

1. *Kept at room temperature.* No. 19.

Material was tested at 4½ months, at 12 to 13 months, and 24 to 27 months.

Age 4½ months. A 2.5 cc. daily dose gave full protection showing little or no deterioration to have taken place.

Age 12 to 13 months. Both 2.5 and 5 cc. daily doses gave protection, showing that after a year at room temperature, little deterioration had still taken place.

Age 24 to 27 months. The material used in this test had not been kept for the whole time at room temperature, for the first 10 months it was kept at about 0°, later being kept at room temperature. 2.5 cc. failed to give protection. 5 cc. dose gave good health up to 60 days and promised to give full protection though the experiment was incomplete at time of publication.

Even after 10 months at about 0° and 14 to 17 months at room temperature, therefore, the loss in anti-scorbutic value of lemon juice preserved with the

rind oil though perceptible was not very great, and the result compares exceedingly favourably with the lemon juices preserved with sulphite, where only one specimen gave a similar result while other specimens kept for shorter periods failed to protect in larger doses.

2. *Material stored in hot room, 37°. No. 20.*

Under these conditions the juice darkened after a few weeks, but smelt and tasted good after many months. The first samples were not tested till after 24 months' keeping. Two animals received a 2.5 cc. dose, two a 5 cc. dose and one a 10 cc. dose. All showed severe scurvy and there was little if any sign of protection even in the case of the 10 cc. dose. Further experiments with this material were therefore abandoned as it was evident that the method of preservation was useless at such a temperature as 37°.

3. *Material stored in cold room ("chilled"; about 0°). No. 21.*

The material was tested for the first time after 26 months' keeping. Two animals receiving 2.5 cc. daily remained in good health for the full period of experiment. Lemon juice with rind oil preserved at about 0° does not therefore lose a perceptible amount of its anti-scorbutic potency even when so stored for more than two years, a property which it shares with the lemon juice preserved with sulphite when that is stored at the same temperature.

Summary of results with lemon juice preserved with the rind oil.

The juice preserved in this way at 0° and 37° gives much the same result as juice preserved with sulphite. Both show little or no loss of anti-scorbutic potency even after two years at 0° and both scarcely appear to retain any potency after preservation for two years at 37°.

. It is in the results at room temperature that the material preserved with rind oil shows its superiority over that preserved with sulphite. In one case, No. 15, the sulphite material appears to be as good, but it evidently cannot be relied upon, particularly after the bottle is opened. There is no evidence that opening the bottle has any destructive effect upon the rind oil material.

(b) *Orange juice with the rind oil.*

The preparation of the material has already been described under the determination of the minimum dose. A portion of the material then tested was set aside for keeping and was preserved at room temperature and at 37°.

Whenever a test was started the portion of material required was kept at about 0° until after the test was finished.

1. *Preserved at room temperature. No. 22.*

Aged 16 to 19 months. This material was not tested till it was 16 to 19 months old.

Of two animals on a 3 cc. dose one developed scurvy towards the end of the 90 days experiment, the other died from other causes at 63 days without signs of scurvy.

On a 5 cc. dose two animals were in good health after 50 days and one after 22 days, the experiment was unfinished at time of publication but promised well and seemed to suggest that orange juice with the rind oil behaves much in the same way as does lemon juice.

2. *Preserved at 37°. No. 23.*

Aged 16 to 17 months. Doses of 2.5, 5 and 10 cc. None of these afforded any protection.

Orange juice preserved with the rind oil does not therefore appear to retain its anti-scorbutic potency at 37° any better than does lemon juice preserved in the same way or with sulphite.

III. PRESERVATION OF CHILLED FRUIT, ORANGES AND LEMONS.

At the request of the Food Investigation Board, some tests have been carried out in order to determine the extent to which the anti-scorbutic principle is retained when fruit is preserved by the chilling process, *i.e.* cold storage at temperatures which do not permit of freezing.

Carefully selected samples of oranges and lemons have been kept in cold storage at a temperature of 2.5 to 5.4°. The fruits were wrapped in paper to prevent contact with one another and packed in crates. They were examined periodically and any found to be soft or mouldy were removed. Fluctuations in the temperature of the storage chamber were registered daily by means of a self-recording apparatus. The highest maximum temperature recorded during the period of storage was 5.4° and the lowest minimum was 2.5°.

After periods of several months, a supply of fruit was removed for testing. The juice was prepared as described for fresh fruit every other day, and the fruit and juice were kept in the Lister Institute refrigerator during the period of experiment.

A. *Oranges.* No. 24. The fruit was put into storage in May 1919 and tests were begun in October, 1919, after five months' storage. At the periodic examinations between these dates, 44 % of the original fruit had been rejected. When the testing was begun 96 oranges were available of which only 11 were perfectly sound; all the rest were partly bad but their juice was tested.

Experiments were made with 5 cc., 3 cc. and 1.5 cc. daily doses and were continued for 75 days only. Two guinea-pigs were used for each test. With all three doses complete protection from scurvy was obtained.

After five months' cold storage of the fruit therefore, the anti-scorbutic potency of orange juice is not appreciably impaired, since a daily dose of 1.5 cc. will still protect a guinea-pig from scurvy (minimum dose of fresh orange juice 1.5 cc. daily, see p. 90).

B. *Lemons*. No. 25. The lemons were first tested in January 1920, after nine months' cold storage. During this period they became very mouldy and at different times 108 out of the original 238 were discarded. At the time of testing none was completely sound.

Experiments were made with 1.5 cc., 2.5 cc. and 5 cc. daily doses. With the 1.5 cc. daily dose severe scurvy terminated the experiment after 32 days. The 2.5 cc. daily dose gave a similar result. Of two animals receiving 5 cc. daily, one died from lung haemorrhage after 28 days, distinct scurvy being present; the other animal showed sore and painful joints by the 25th day and died from severe scurvy after 51 days.

Thus even the 5 cc. daily dose was practically useless, whereas in the fresh state a 1.5 cc. daily dose of lemon juice affords complete protection from scurvy. The test is not satisfactory because the fruit was not prevented from becoming mouldy. It was in much worse condition and four months older than were the oranges, though neither was in a condition which would ordinarily be regarded as edible. It is probably therefore safe to say that the juice of oranges and lemons, which are preserved by chilling, does not lose appreciably in anti-scorbutic value as long as the fruit is fit for food, but it cannot apparently be kept fit for food in a "chilling" cold store for more than a very few months.

CONCLUSIONS AND SUMMARY.

1. Further experimental evidence is advanced as to the inferiority of lime juice to lemon and orange juice as an anti-scorbutic.

2. The minimum daily doses of the juice of these three Citrus fruits, needed to protect a guinea-pig from scurvy are established as follows:

Lemon	1.5 cc.
Orange	1.5 cc.
Lime	5.0 cc.

These values are used as a basis for comparing the keeping properties, as anti-scorbutics, of the juice of oranges and lemons. The juice is kept for varying times at different temperatures, and in the case of lemon juice preservation with sulphite and with the rind oil is tested, and in the case of orange juice preservation with the rind oil.

3. Preservation with sulphite appears to be satisfactory at a low temperature (about 0°) but at room temperature it seems uncertain and at 37° it is useless, the juice becoming bad in the gross sense as well.

4. Preservation with the rind oil, in case of lemon, is satisfactory and reliable at about 0° and at room temperature. With oranges preservation by this method at 0° was not tried but at room temperature the result was satisfactory. At 37°, both for oranges and lemons, it is as unsatisfactory as is preservation with sulphite, though the juice was not as unpalatable as was the juice preserved with sulphite.

Consequently for 0° and for temperatures about English room temperature, preservation with the rind oil is suggested as the most reliable. There is loss, but even after two years it is not very great.

5. Preservation at 37° has not so far been secured by any method; it is possible that could preservation in the gross sense be secured, the anti-scorbutic property might also be better preserved; it is suggested that this might be attained by boiling the juice first to render it sterile. It has been shown by Delf [1920] that to heat orange juice for an hour at 100° does not materially diminish its anti-scorbutic potency.

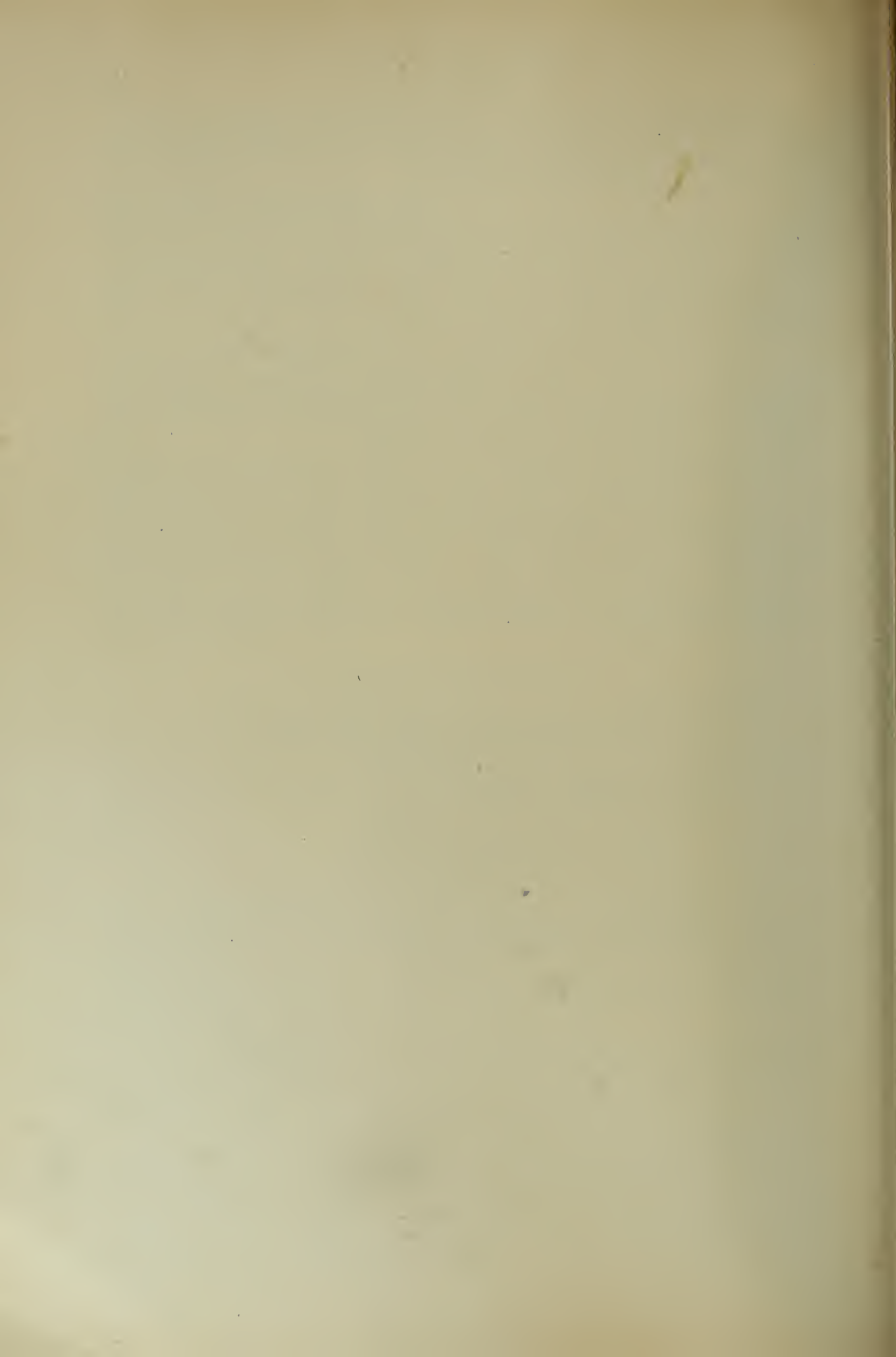
6. Experiments are also described on the preservation of oranges and lemons, kept in a chilling (not freezing) cold store; the results are not quite consistent but it appears probable that the anti-scorbutic property is not seriously diminished so long as the fruit is edible.

In conclusion it is my pleasant duty to record my gratitude to Miss Hume, who when I was obliged to resume my university work, undertook the task of final preparation of this paper for the press. Thanks are also due to Miss H. Henderson Smith and to Miss S. Rutherford for assistance in feeding the animals.

A part of the cost of the research was defrayed by the Medical Research Council to whom thanks are also tendered.

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LXXII. THE NUTRITIVE VALUE OF LARD.

BY JACK CECIL DRUMMOND, JOHN GOLDING, SYLVESTER SOLOMON ZILVA AND KATHARINE HOPE COWARD.

From the Institute of Physiology, University College, London, the Research Institute in Dairying, Reading, and the Biochemical Department, Lister Institute, London.

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With Plate XII.

DURING the last few years a considerable amount of attention has been devoted to the study of the distribution of the so-called fat-soluble accessory factor, or vitamin A, in naturally occurring oils and fats. The results of these investigations tend to show that the oils and fats derived from the animal kingdom are, as a rule, decidedly richer sources of this essential dietary constituent than those prepared from vegetable sources. Most authorities, however, regard lard as an exception, having found it practically devoid of vitamin A. Quite early in the study of the growth-promoting vitamins McCollum and Davis [1913] observed that butter fat is of much higher nutritive value for growth than lard.

A similar conclusion was reached by Osborne and Mendel [1913], who suggested that the difference might be ascribed to the fact that lard is a fat derived from storage depôts, whereas butter fat is a product of the synthetic processes of the mammary gland. Many later workers have confirmed these experimental results, and lard has usually been regarded as of little or no value as a source of the fat-soluble A factor.

When one considers the enormous quantities of lard which are prepared for edible purposes at the present time, the importance of ascertaining why lard is thus deficient will be realised. So far as we are aware, no systematic attempt to solve this problem has been made; and only one reference bearing on this question has been encountered in the literature.

Osborne and Mendel [1915] refer to one experiment which they carried out in order to ascertain whether the inefficiency of lard is due to the technical processes to which the fat of pigs is subjected in preparation for the market. Pig fat, direct from the slaughter-house, was finely divided and filtered through filter paper at a temperature just above its melting-point. The filtered product, which they termed "Laboratory Lard," was found to be as inadequate for growth as the commercial products. This result led the authors to conclude that the inferior nutritive value of lard is not due to the heating

which the fat may have received in the course of preparation. This experiment must, however, be regarded as of little value, since apparently no test of the growth-promoting power of the unheated pig fat was made.

In view of the very great importance of the question of the food value of lard, we decided to subject the matter to an experimental study, particularly since it had been observed by Drummond and Coward [1920, 1] that some specimens of raw pig fat contain appreciable amounts of the vitamin A.

Since it is now experimentally proven that the mammalian organism does not possess the power to synthesise the vitamin A, and that it is dependent on its diet for supplies of this essential factor, we concluded that the investigation must proceed along two lines, first, a study of the influence of the diet of the pigs on storage of the vitamin in the fat depôts, and secondly, an investigation of the influence of the technical processes of lard manufacture on the vitamin when present in the pig fat.

The first series of experiments was carried out at the farm attached to University College, Reading, and we desire to thank Prof. Pennington, the Director of the farm, for granting us facilities for the work. The rat feeding tests were carried out at University College, London.

I. INFLUENCE OF DIET OF PIGS ON THE VITAMIN CONTENT OF PIG FAT.

Experimental.

A litter of Berkshire pigs farrowed in the early part of 1920 was selected for this experiment. With the exception of one pig, which was a weakling, all the animals were strong and healthy. They were permitted to remain with the sow until weaned at the age of eight weeks, when they were given a ration of toppings (wheat pollards) and whey.

At the age of nine and a half weeks they were divided into five groups. Groups I-IV, which were to be placed on controlled diets, each contained a hog and a sow, whilst Group V, which were the real controls, contained two hogs.

Groups I and II were kept in large, well-designed, stone-floored experimental styres, since it was intended to give them a diet deficient in vitamin A. Groups III and IV were kept in a form of moveable pen improvised by one of us, which permitted the animals to have access to a new plot of green pasturage every day. Group V were reared in the usual manner employed on this farm, and not only received a mixed diet, but also had free run of a small grass paddock.

Group I were given a seriously deficient diet, namely one of toppings and a "synthetic whey." This latter constituent was prepared from caseinogen, lactose, olive oil and salt mixture so as to represent the composition of the whey used in the other experiments, as determined by frequent analysis.

Experiments on rats indicated that this diet was almost devoid of all three



1. Side view.



2. Front view.

Photographs of the slaughtered pigs.

From left to right

Group	Sex	Diet	Weight lbs
II	♂	Toppings, whey	122
IV	,,	Toppings, grass	103
V	,,	Full diet with grass	150
V	,,	“ “	—
III	,,	Toppings, whey and grass	120
I	,,	Toppings, synthetic whey	84



vitamins A, B and C. It was not, therefore, considered likely that the pigs would thrive at all on the food mixture. To our astonishment both animals showed excellent increments of weight for a considerable period of time.

Fig. 1 shows the growth of the hog of this series. It will be seen that the initial growth is considerable in spite of the deficient nature of the ration. Ultimately the animals in this group showed retardation of growth, and lost their healthy appearance. We do not, however, intend to discuss this side of the experiments at this point since we have made a number of other observations on the growth of pigs on deficient diets which will form the subject of a later communication.

Group II were fed on a food mixture of similar composition to that employed for Group I, but the whey was the natural product. Tests of the whey made on rats indicated that it contained insignificant traces of the factor A.

Group III received a basal diet identical with that given to Group II, namely whey and toppings, but in addition were allowed to have an unlimited supply of fresh green food, an addition which they welcomed and of which they made good use.

Group IV received no whey, and were confined to a diet of toppings and green foods. Group V received a well-balanced and varied farm diet, including ample grass.

Growth curves of one animal (hog) from each group are given in Figs. 1-5, but, as we have remarked above, we do not intend to discuss this side of the investigation here. Records of food intake were made daily in the case of Groups I-IV, and frequent analyses of the toppings and whey were carried out to control the intake of nutrients. Attached to the growth curves are diagrams indicating the average daily food consumptions at various periods of the experiments.

At the end of nearly three months on the experimental diets, the condition of the hogs on the deficient diets 1 and 2 and the size of the control animals led us to conclude that a suitable point had been reached for testing the body fats for storage of the factor A. In view of the fact that our material was limited, we decided to slaughter the hogs of each group only, retaining the sows for the purpose of other observations which we desired to make. Accordingly this plan was carried out.

The hogs were sent to the butcher 24 hours before being slaughtered. A post-mortem examination did not reveal any noticeable abnormality in the organs of the animals. After the carcasses had been "dressed" in the usual manner, samples of back fat and perinephritic fat (fleck or leaf) were removed. Photographs of the dressed carcasses are given which show the relative sizes of the animals from each group (Plate XII). The small size of the hog fed on the deficient diet given to Group I is obvious, as are the well-developed bodies of the two animals in the control Groups III and V, especially in the latter.

The fats were tested for the presence of the factor A by observing the

influence of daily supplements of known weight on the growth of rats, whose growth had been brought to a standstill by a deficiency of that substance.

Small cubes of the fat weighing approximately 1.5 g. were administered to each rat before the daily ration of the basal diet devoid of the factor A

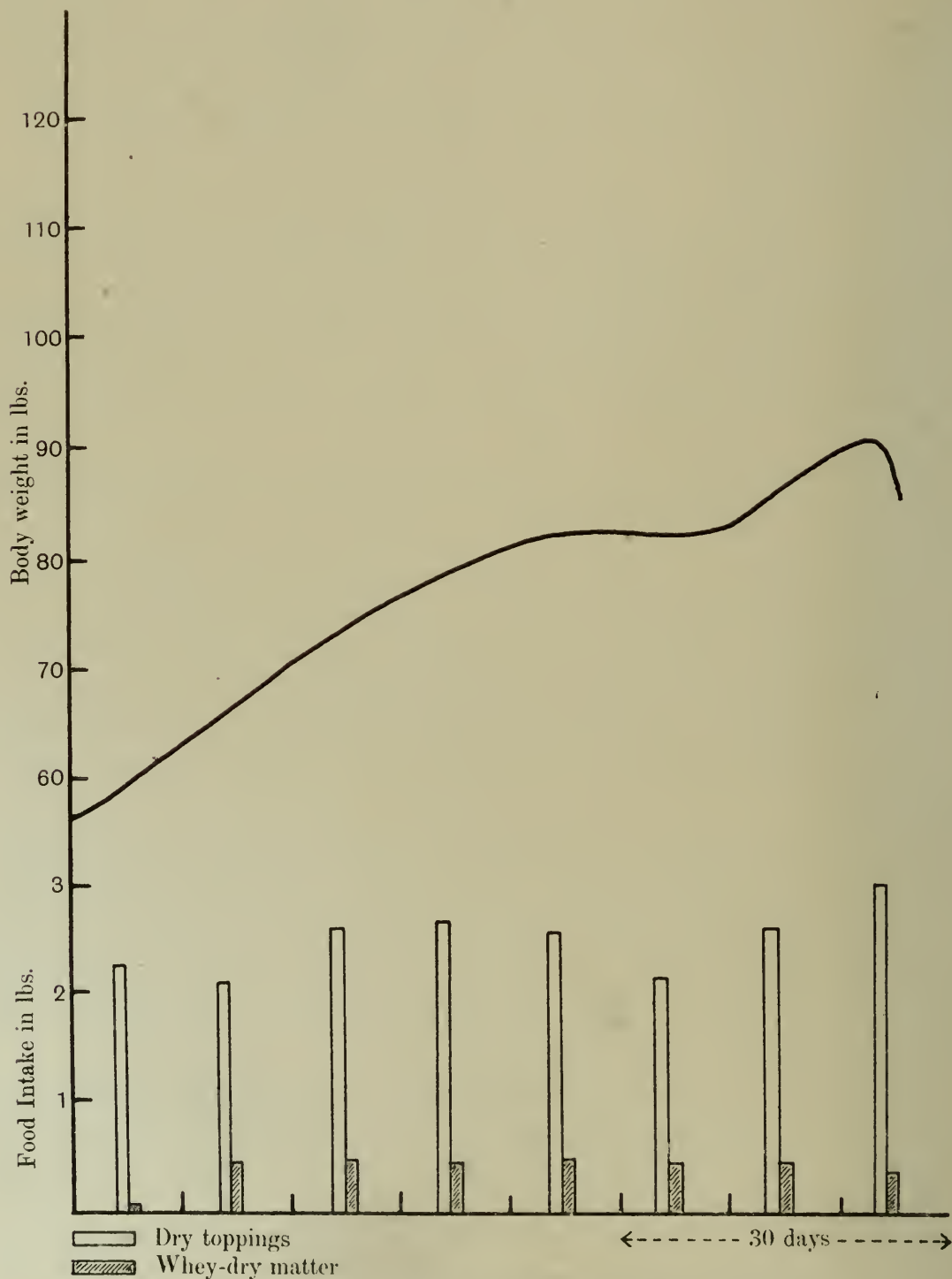


Fig. 1. Growth curve and food intake of hog from Group I.

was given. Practically without exception the whole of the supplements were consumed. The results of these feeding tests are in our opinion quite striking. The body fats derived from pigs in Groups I and II fed on diets deficient in

the vitamin A were found to be of no value as sources of that essential substance for the rat (Curves 1-11, Fig. 6). On the other hand, there is marked evidence that the body fats of the grass-fed animals, particularly those

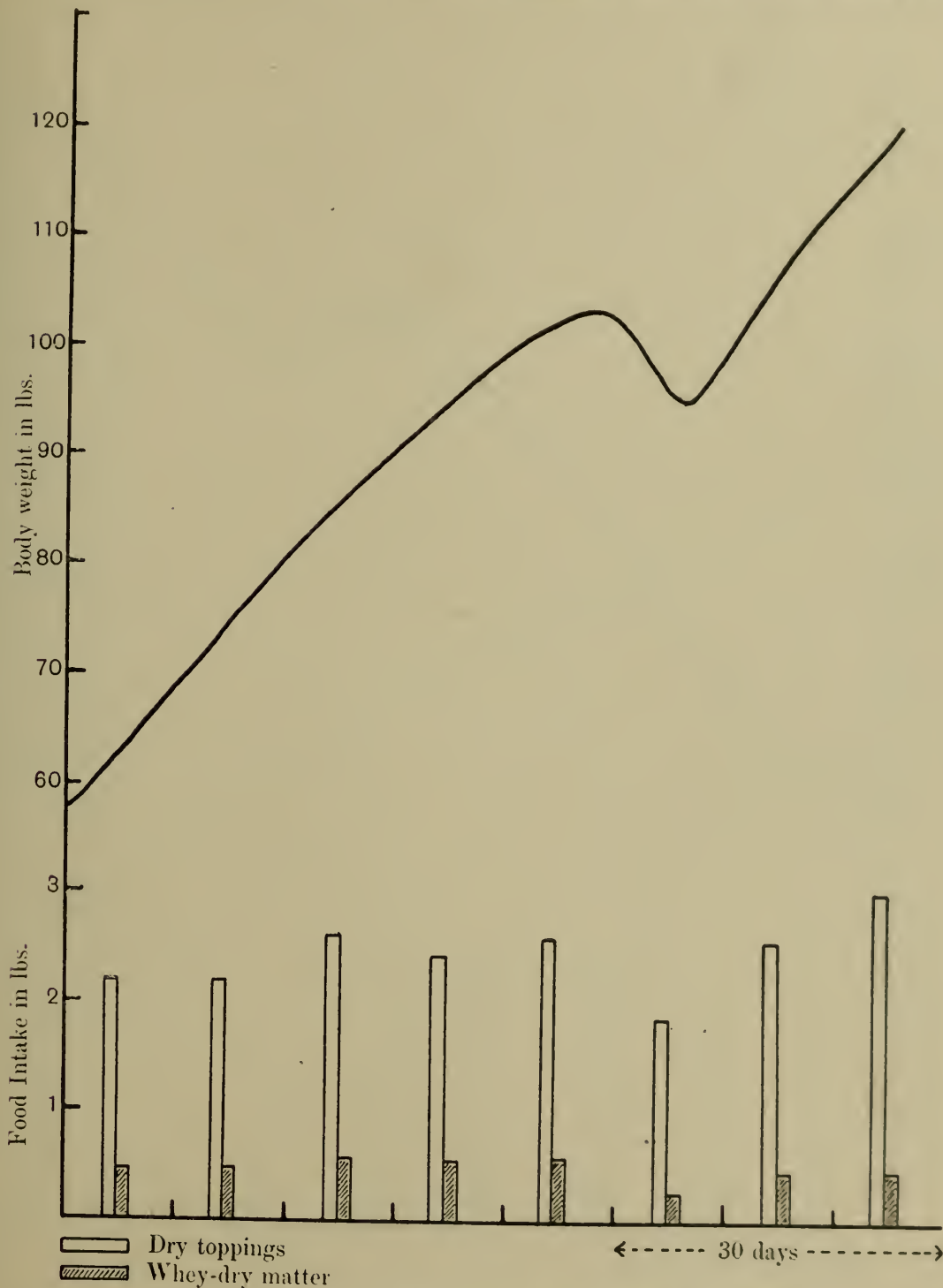


Fig. 2. Growth curve and food intake of hog from Group II.

receiving a mixed farm ration in Group V contained appreciable amounts of the important growth-promoting accessory substance (Curves 12-28, Fig. 6).

The fats from the abdominal cavity and from the subcutaneous deposits were tested separately in each case.

NUTRITIVE VALUE OF LARD

These experiments demonstrate that storage of the vitamin A will occur in the body fat of the pig, provided that the animal receives a diet containing considerable amounts of that substance. This finding is of importance since

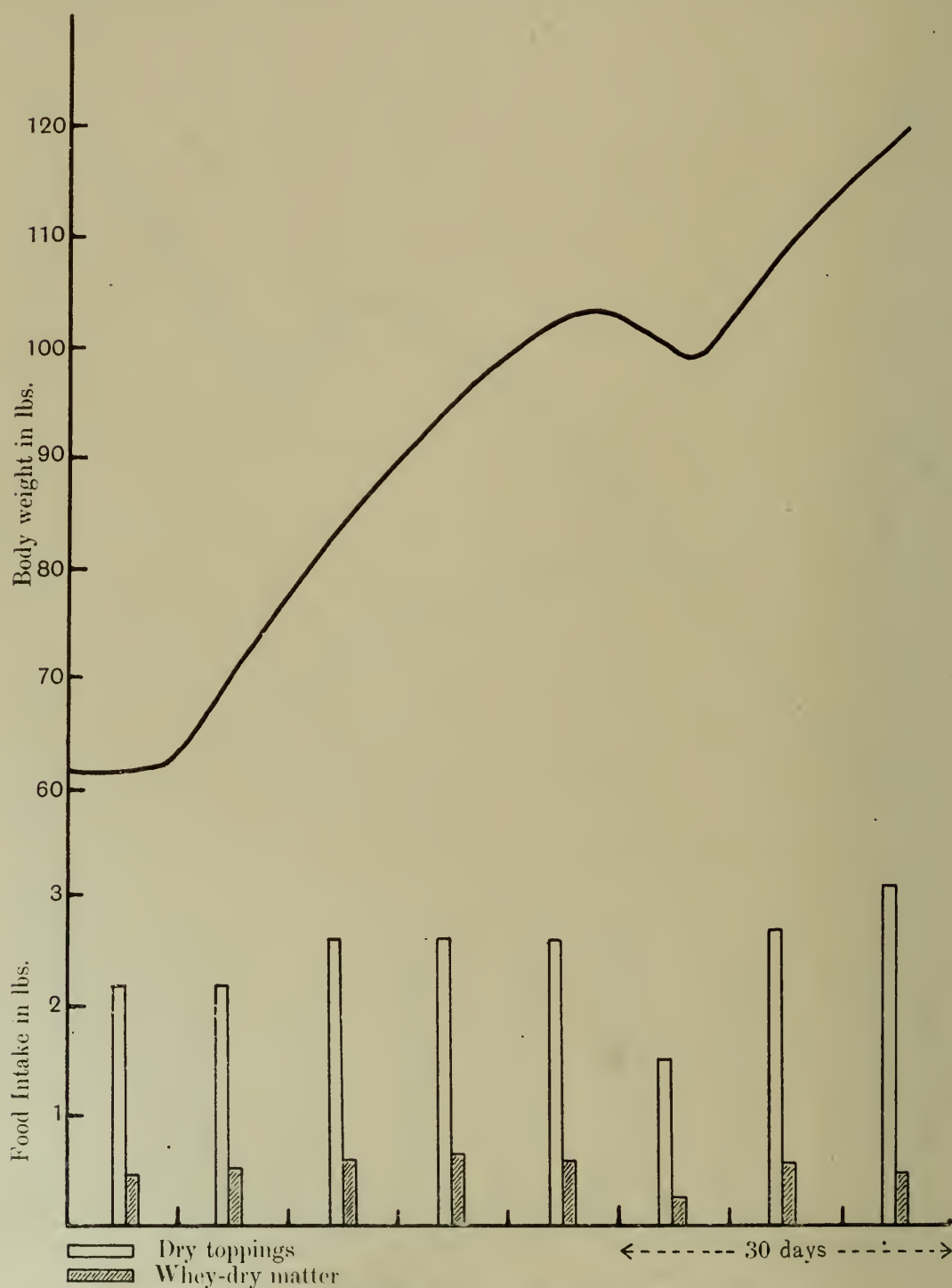


Fig. 3. Growth curve and food intake of hog from Group III.

it shows that the pig is not an exception to the rule that storage of the fat-soluble vitamin A in the fat depôts occurs in animals under suitable conditions.

It does not appear that pig fat is, weight for weight, as rich in vitamin A as is the body fat of other animals fed on a similar type of diet. This may,

however, be due to the fact that the mass of adipose tissue is so much greater in the former species, and that the concentration of vitamin per unit weight of fat would tend to be smaller than in a species such as the cow, where fat deposition is less marked.

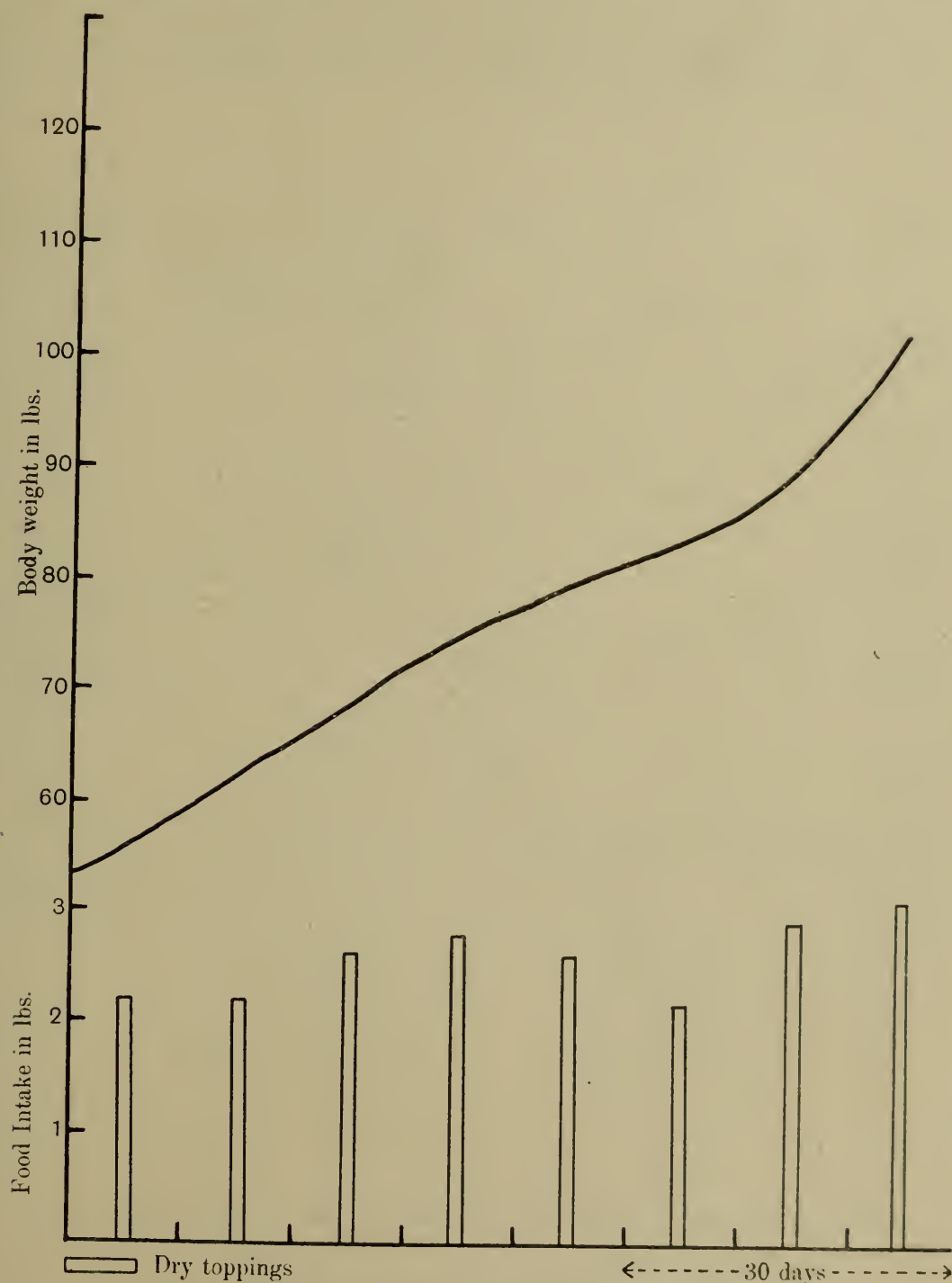


Fig. 4. Growth curve and food intake of hog from Group IV.

II. INFLUENCE OF THE PROCESSES EMPLOYED IN THE MANUFACTURE OF LARD ON THE VITAMIN PRESENT IN PIG FAT.

The enormous quantities of pig fat that are converted into lard for human consumption every year may be judged from the fact that in 1912 the total

weight of lard exported from the United States was over 500 million lbs. A considerable proportion of this production is utilised for margarine manufacture, an industry which has grown to many times the size it was in 1912. It is therefore of very great importance to ascertain the influence of the method of preparation employed in lard manufacture on the nutritive value of the fat. Having found by the feeding experiments described above that

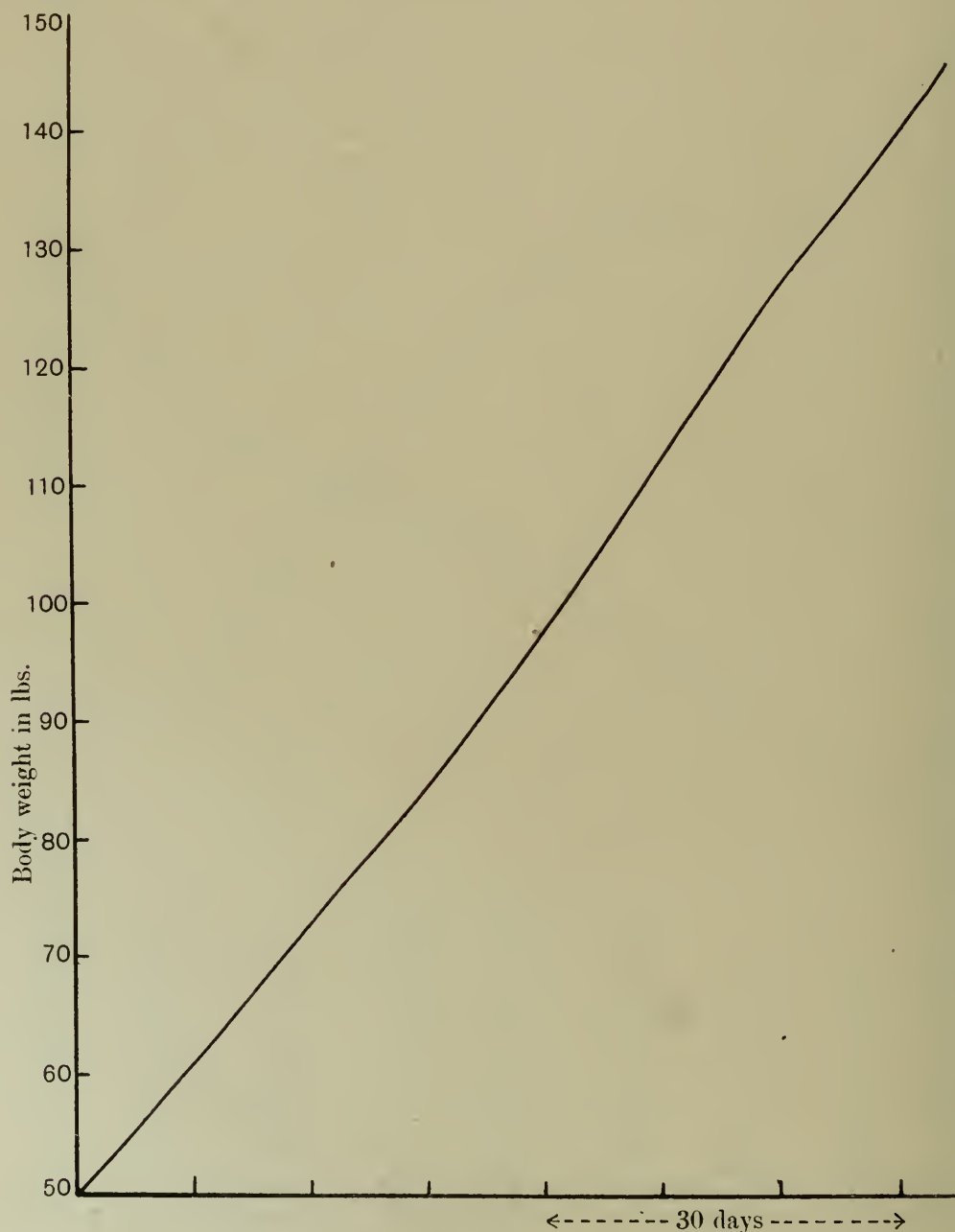


Fig. 5. Growth curve of hog from Group V.

pig fat may contain appreciable quantities of vitamin A when the pigs have been fed on a diet rich in that factor, we proceeded to investigate how the accessory factor is affected by the processes of lard manufacture and refinement. Lard manufacture in this country is not carried out on anything approaching the enormous scale that may be seen in America, and there are

distinct differences between the methods employed there and in this country. The oldest and simplest method of lard preparation still survives in the country parts of England, and we had the opportunity of seeing one preparation by this method. It is only employed by local pig butchers or farmers who handle small numbers of swine, and the amount prepared in this manner is negligible when one considers the total production of lard; the product is largely used for consumption in the immediate locality. This process consists

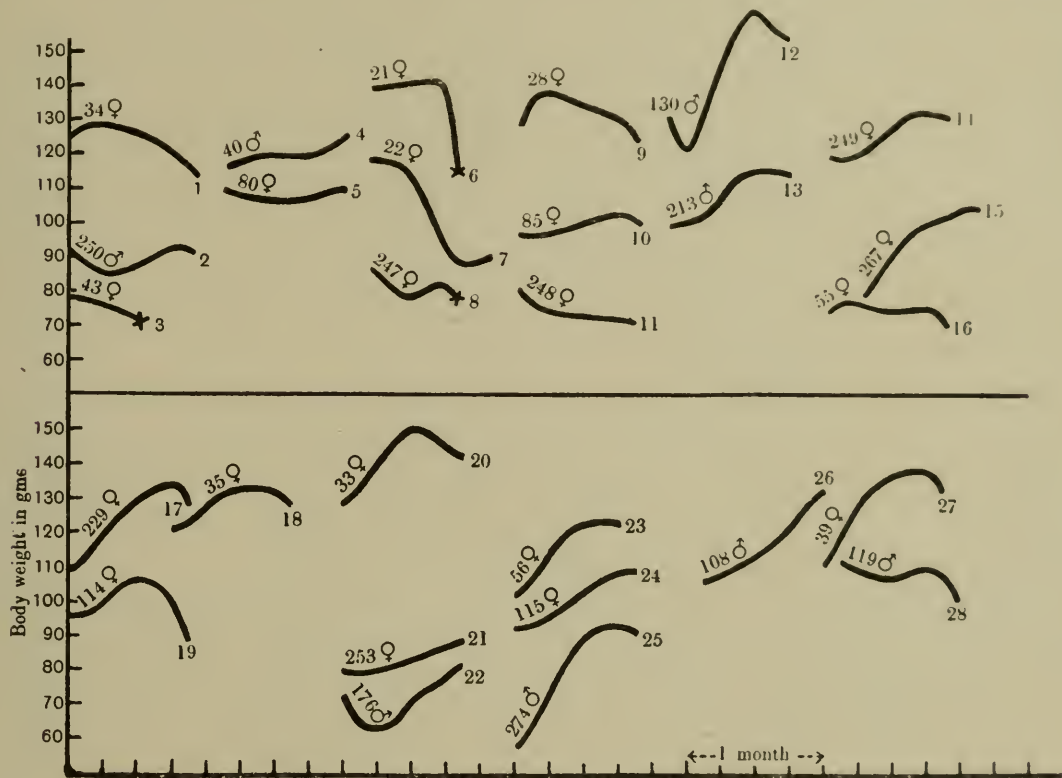


Fig. 6. Growth curves of rats whose diets were supplemented by a daily ration of 1.5 g. fat from the slaughtered pigs.

- | | |
|----------------------------------|---------------------------------------|
| Rats 1-3 Group I (back fat); | Rats 14-16 Group III (abdominal fat); |
| „ 4-5 Group I (abdominal fat); | „ 17-19 Group IV (back fat); |
| „ 6-8 Group II (back fat); | „ 20-22 Group IV (abdominal fat) |
| „ 9-11 Group II (abdominal fat); | „ 23-25 Group V (back fat); |
| „ 12-13 Group III (back fat); | „ 26-28 Group V (abdominal fat). |

The preliminary period of inhibited growth on the basal ration is omitted.

in heating the pig fat in an open pan over a fire, stirring meanwhile. The melted fat is from time to time skimmed from the surface, reheated to drive off moisture, strained, and poured into bladders. Lard prepared in this manner usually has a brownish tint due to admixture with products derived from the charred tissue. In view of the very small amount of lard which is prepared by this simple farmhouse method, we decided that it was unnecessary to investigate its food value.

For permission to view a large modern lard manufactory in this country we are indebted to Mr R. J. Harris, of the firm of Harris and Co., Bacon Curers, Calne, Wiltshire. To him, and to Mr O. Jones, the chief chemist at

that factory, we wish to express our appreciation of the very kind manner in which they assisted us in our investigation, by placing much information and numerous samples at our disposal. The method employed in this factory is essentially as follows:

The pigs which are drawn from a large area of the surrounding country are slaughtered and the carcasses dressed in the usual manner. When cold, the abdominal fat or fleck, which has been stripped and hung up beside the carcass, is minced in a large mechanical mill. The fatty pulp passes into a steam-jacketed pan provided with stirrers, where it is heated to a temperature of about 82°. The fat which separates from the tissues runs off into a second steam-heated pan, where heating at a temperature just above boiling-point (102°) is maintained with stirring for about 10 or 15 minutes to drive off any moisture. The hot dehydrated fat is then clarified either by passing it through a filter press or by allowing it to stand in a settling tank. The lard is now ready to be converted into a suitable solid form and packed for the market. The details of these latter processes have no bearing on the subject of this paper and are therefore omitted.

Very little pig fat other than the perinephritic deposits is used for lard making in this factory, and consequently the product is one of very high standard. The dimensions of the lard industry in the United States have necessitated the adoption of a system of classifying lards. Many of the preparations are made from inferior sources of fat.

The Rules of the Chicago Board of Trade define the following brands of edible lard. (a) Neutral Lard No. 1, which is a high quality lard prepared only from the abdominal leaf. (b) Neutral Lard No. 2, a similar product prepared from the back fat. These two types are carefully prepared and since they have not been "cooked," they do not keep well. Their chief use is in the manufacture of high class margarines. Only a small amount of lard is prepared by this method in this country. (c) Leaf Lard. This is essentially a product which has been prepared by a process similar to the one we have described in detail above. (d) Choice Lard or Kettle-rendered Lard, and (e) Prime Steamed Lard are products of much lower standard. The latter is indeed usually prepared from the trimmings of the carcasses and not from the true fat deposits. They are frequently rendered by the use of high pressure steam, and since they are unpalatable and discoloured in the crude state, they are usually treated by "blowing," or some such process involving oxidation, in order to remove colouring substances and a somewhat unpleasant odour and taste.

We began our investigation by examining an average sample of the pig fat such as is used at Calne for lard manufacture. Curves 4-6, Fig. 7 show that this sample contained an appreciable amount of the fat-soluble vitamin. This confirms the observations by Drummond and Coward [1920, 1] that pig fat may contain the factor A. We also tested a sample of lard which had been made from the same batch of pig fat. This proved to be inactive (Curves 1-3,

Fig. 7) as did several other samples of lard made there on other occasions. Apparently the processes of manufacture were responsible in some manner for the removal or destruction of the vitamin. Naturally the inactivation of the vitamin present in the pig fat will depend on the amount present, which will presumably depend to some extent on the age of the pigs when slaughtered, and to a greater extent on the diet upon which they had been reared.

It was impossible to obtain precise information regarding the diet which had been given to the pigs from which the samples of fat ultimately made into lard were taken. We were informed, however, that it is general in the areas from which the majority of the pigs are drawn to supplement the usual type of diet of toppings, barley meal, whey, etc. with kitchen refuse and green foods. Grass feeding of swine is not carried out to any large extent in this country.

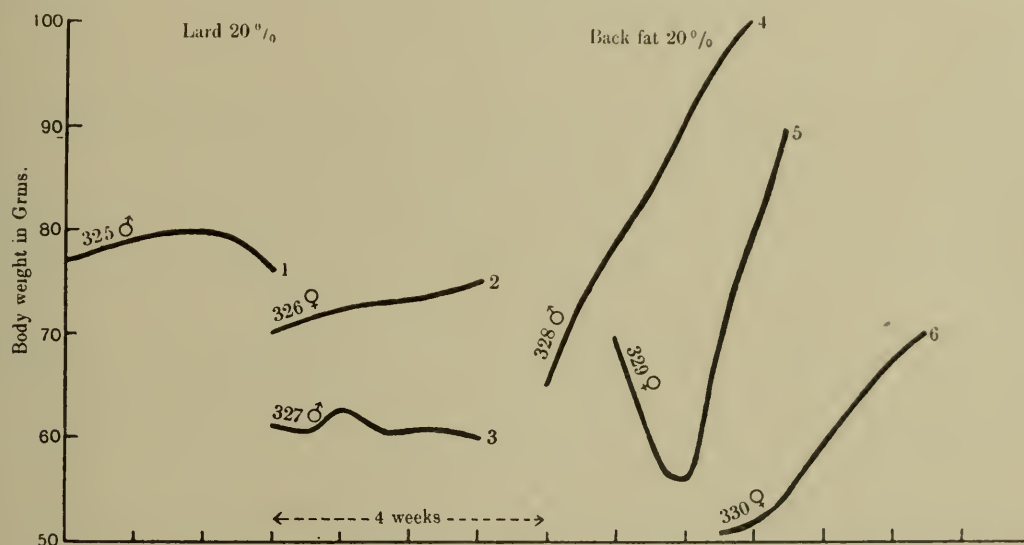


Fig. 7. Growth curves of rats whose diets contained 20 % lard (1-3) and back fat (4-6) respectively.

The preliminary period of inhibited growth on the basal ration is omitted.

The cause of the loss of vitamin during lard manufacture is in our opinion largely due to oxidation, since it has recently been shown that the factor is rapidly inactivated at high temperatures by air or oxygen or by ozone in the cold [Hopkins, 1920, 1, 2; Drummond and Coward, 1920, 2; Zilva, 1920].

The concentration of vitamin A in pig fat, even in grass-fed animals, appears to be considerably lower than in fat derived from grass-fed cattle, and much lower than that usually found in butter, so that when it is remembered that butter fat exposed over a large surface to air at temperatures about 100° may be inactivated in a time as short as one or two hours, it can be understood how the pig fat loses its accompanying vitamin during the heating and stirring used in its conversion into lard.

Recently a paper has appeared in which Daniels and Loughlin [1920] claim to have examined samples of lard which showed considerable growth-promoting power. Not only good growth, but reproduction and satisfactory

rearing of the young were accomplished by their rats when their diet contained 28 % of a commercial preparation of lard. It is possible that the sample with which they worked was one prepared from a pig fat of very rich vitamin content, and that appreciable amounts of that factor had escaped destruction.

Since this communication was completed a sample of lard prepared at Calne from some very active back fat has been examined by us and has been found to possess some activity although not nearly as pronounced as that observed by Daniels and Loughlin. This suggests that some of the fat-soluble vitamin may remain in the lard after treatment in certain cases.

SUMMARY.

1. The pig is able to store up supplies of vitamin A in the body fat when fed upon a diet containing ample supplies of that factor, as for example when grass-fed.

2. When the diet of the pig is deficient in vitamin A, as for example when the diet consists almost entirely of toppings and whey, no appreciable amounts of that dietary factor can be detected in the body fat.

3. The processes employed in the manufacture of lard on a large scale in this country cause a very marked destruction of the vitamin present in the pig fat.

4. The low nutritive value of lard is therefore believed to be due to two causes. First, the diet usually given to fattening pigs in this country is seldom rich in vitamin A, so that the average sample of pig fat contains little or none of that substance; secondly, the processes of lard manufacture undoubtedly cause the destruction of much of the vitamin present in the original pig fat, probably owing to the exposure of the fat to oxygen at high temperature.

The expenses of this research were defrayed from a grant made by the Medical Research Council, to whom our thanks are due.

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No 6

LVII. THE EFFECT OF PYRUVATES, ALDEHYDES AND METHYLENE BLUE ON THE FERMENTATION OF GLUCOSE BY YEAST JUICE AND ZYMIN IN PRESENCE OF PHOSPHATE.

BY ARTHUR HARDEN AND FRANCIS ROBERT HENLEY.

From the Biochemical Department, Lister Institute.

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OPPENHEIMER [1915] observed that the fermentation of glucose by maceration extract was greatly stimulated by the addition of a pyruvate or pyruvic acid and that acetaldehyde had a similar but less pronounced effect. The estimations were made by weighing at comparatively long intervals but it is obvious in the case of acetaldehyde, that the stimulation chiefly occurs at the commencement of the fermentation. The results with pyruvates are not always so clear and are apparently complicated by some unconsidered factor since in some cases the total CO_2 evolved exceeds that obtainable from the glucose and pyruvate in the normal course of fermentation (e.g. Br₁, Table III, p. 240 in which 316 milligrams of CO_2 are obtained from 0.4 g. of glucose + 0.04 g. pyruvic acid which would together only yield in the normal course 220 mgms. or including the observed autofermentation 230 mgms., and Br₂ in the same table where 295 mgms. are obtained against 212 normally obtainable).

Neuberg somewhat later [1915] observed a similar stimulating action of pyruvates and other α -ketoacids on the fermentation of glucose, mannose, fructose and saccharose and remarked that the activation was most pronounced at the commencement of the fermentation. Experiments continued for 19–20 hours (p. 82) showed little difference in the total fermentation in the presence and absence of pyruvate.

Neuberg subsequently examined the effect of a large number of aldehydes [1918] on alcoholic fermentation and found that they were all vigorous activators. He pointed out that the effect was most marked with glucose and mannose, less so with fructose and cane sugar and suggested that this fact might be related to the observation of Harden and Young [1909] that fructose under certain circumstances can stimulate the fermentation of glucose. The stimulation, like that produced by pyruvate, was most marked at the commencement of fermentation.

Meyerhof [1918] has made an interesting contribution to this subject in his study of the kinetics of cell-free fermentation. Lebedev [1912] observed that when maceration extract was mixed with a fermentable sugar a period of *induction* occurred during which no CO_2 was formed and no change in rotation occurred, and this has frequently been confirmed. Meyerhof now finds that this induction is not observed when the extract contains even a trace (0.2 millimolecular concentration) of a hexosephosphate and hence has never been recorded for juice prepared by Buchner's process, in which recognisable amounts of hexosephosphate are present. The period is moreover shorter in presence of cane sugar than of fructose or glucose and is diminished when these two sugars are warmed for 4–6 hours at 80° with a neutral phosphate mixture. The induction period is also greatly lessened by grinding the dried yeast with glass powder before maceration.

Following on the induction period, the velocity of fermentation more or less gradually attains a maximum corresponding to the concentration of phosphate present. This is termed by Meyerhof the "Gäranstieg" and has been studied in some detail. The effects of the addition of excess of phosphate described by Harden and Young [1908], viz. a diminished maximum and a more gradual attainment of the maximum¹, are found by Meyerhof to be also produced by the addition of salts such as sodium chloride or nitrate and hence the phenomenon is partly to be explained as a general salt effect. The rate of attainment of the maximum becomes greater as the hexosephosphate concentration increases, but this characteristic phenomenon cannot entirely be abolished by the addition of hexosephosphate. Meyerhof discusses the cause of the phenomenon and shows by an ingenious application of the effect of arsenates that it is probably not due to the production of a specially labile form of sugar from the hexosephosphate. The attainment of the maximum was also found to be more rapid the greater the concentration of the coenzyme, added in the form of boiled extract of yeast or muscle.

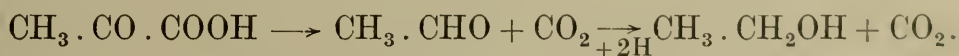
The facts that the activating effect both of α -ketoacids and of aldehydes was chiefly manifested at the commencement of the reaction, that the experiments both of Oppenheimer and Neuberg were made with maceration extract which contains a large amount of mineral phosphate and that the effect was less marked with fructose than with glucose led us to enquire whether this action was a general stimulation of the fermentation process or a more specific acceleration of the reaction in presence of free mineral phosphate. The results show that when aldehyde is added to fermenting mixtures of yeast juice or

¹ Meyerhof [1918, p. 196] erroneously attributes to Harden and Young in explanation of this phenomenon the suggestion that the *sugar* forms with high and low concentrations of phosphate different esters of different stability, one of which, as the phosphate is used up, passes into the other. What they actually suggested was [Harden and Young 1908] that the phosphate is capable of forming two or more different unstable associations with the *fermenting complex* (by which was meant the complex of enzymes concerned, not the sugar). The alternative suggestion has also been made [see Harden 1914] "that the addition of increasing amounts of phosphate causes a progressive but reversible change in the mode of dispersion of the colloidal enzyme."

zymin (acetone yeast) with glucose no marked acceleration in the normal rate of fermentation occurs. If a suitable amount of phosphate be then added, sufficient to cause only a gradual rise of rate to the maximum in the control experiment with glucose, the effect of the presence of the aldehyde is greatly to diminish the time required for the attainment of the maximum, so that the volume of gas evolved in the period immediately following the addition of the phosphate is greatly increased. At the same time a considerably higher maximum is attained. On the completion of the esterification of the phosphate, the rate again diminishes both in the presence and absence of aldehyde and the total evolution is not greatly different in the two cases. Similar phenomena are produced by the addition of pyruvates. The effect varies with the concentration of aldehyde and is common, but in unequal measure, to the four aldehydes tested (formic, acetic, propionic and butyric). So far only glucose has been employed as the fermentable sugar, but experiments are in progress with fructose and cane sugar.

This striking effect of aldehydes, which are known to be readily reducible by yeast, strongly suggested that the cause of the delay in attainment of the maximum after the addition of phosphate was lack of an acceptor for hydrogen. In order to test this idea, methylene blue, which is also readily reducible by yeast, was substituted for the aldehyde, with the result that it was found to produce a very similar effect. When increasing amounts of methylene blue are added a point is soon reached at which the maximum, although it is more rapidly attained, is considerably lowered. This is probably due to the inhibitory effect of the dye on the enzyme complex. Even with the most favourable concentration of methylene blue however the rise of rate was not so rapid as with the aldehydes, and the maximum was unchanged.

According to the pyruvic acid theory, which may now be taken as established, the final stage of the alcoholic fermentation of sugar is the reduction of acetaldehyde (produced by the decomposition of pyruvic acid), a reaction which proceeds so rapidly that only an extremely small concentration of the aldehyde is present during normal fermentation.



Further, the production of the pyruvic acid from sugar appears only to be possible when some acceptor for hydrogen is available, this being normally supplied by the acetaldehyde produced in a later stage of the reaction.

On this view it would seem to follow that no rise in the rate of fermentation can occur without the provision of an additional quantity of a hydrogen acceptor. Some such acceptor is probably more or less rapidly formed and reduced during the period of delay, which follows on the addition of phosphate, this process being accompanied by a corresponding increase in the formation of pyruvic acid, until sufficient of this is being produced to provide the amount of acetaldehyde necessary for the maximum effect. When, however, the easily reducible aldehydes or methylene blue are added, these act as

acceptors and a much more rapid or even instantaneous attainment of the maximum becomes possible, as was actually observed in our experiments.

Whether this acceptor is the same substance as yields glycerol in the sulphite fermentation of Neuberg and Reinfurth, and is supposed by Neuberg to be methylglyoxal, is uncertain. It may be pointed out however that the precursor of glycerol assumed by Neuberg and Kerb [1913] must be much less rapidly reduced than acetaldehyde in the course of normal alcoholic fermentation since the ratio of glycerol to alcohol under these circumstances is only small. No experiments have yet been made to decide whether an enhanced glycerol production occurs during the period of delay.

It seems probable that the delay following the addition of phosphate when fructose is employed as the fermentable sugar is also due in part to lack of an acceptor. The facts that fructose yields a much higher maximum rate with phosphate and that the optimum concentration of phosphate is much higher than for glucose can accordingly be interpreted to mean either that fructose yields a hydrogen acceptor much more readily than glucose or that the acceptor formed is much more rapidly reducible. This question is at present under investigation. If this conclusion be granted, a simple explanation is afforded of the remarkable "induction" observed by Harden and Young [1909] when fructose was added to a mixture of yeast juice and glucose or mannose to which a considerable excess of phosphate had been added. Under these circumstances the rate of attainment of the maximum fermentation was greatly accelerated even when the phosphate concentration was kept constant and moreover the volume of CO_2 evolved under these circumstances was much greater than could be obtained from the fructose added. In the light of the foregoing remarks it now appears that the function of the fructose under these conditions is probably to provide a hydrogen acceptor and this, once formed, enables the fermentation of the glucose to proceed rapidly, as explained above, even in the presence of a concentration of phosphate, which in the absence of an acceptor causes a prolonged delay.

It is further probable that the hydrolysis of the hexosephosphate, both that originally present and that slowly formed in the fermenting mixture, results in the formation of fructose, which in its turn yields a hydrogen acceptor and thus assists in the increase of the rate of fermentation. Meyerhof's observations on the marked effect of hexosephosphate on the rate of attainment of the maximum would thus receive a simple explanation.

Owing to the method of experiment employed by us, the full effect of the addition of hexosephosphate could not be observed as the fermenting mixtures always contained this substance formed from the phosphate originally present. The addition of a further quantity of hexosephosphate produced very little effect.

Whether the lack of acceptor combined with Meyerhof's "salt-effect" of the excess of phosphate provides a complete explanation of the delay in

attainment of the maximum after the addition of phosphate or whether time is required for some other change, such as transformation of the sugar into a fermentable form, as maintained by Euler, remains as a subject for further investigation.

EXPERIMENTAL.

The yeast juice and zymine employed were both prepared from a brewery top-yeast. The acetaldehyde used in Exp. 2 was a preparation obtained from Kahlbaum. For the other experiments it was prepared from paraldehyde by distillation with dilute sulphuric acid. The formaldehyde was a dilution of formalin and the concentration was estimated by Ripper's method. The propionic aldehyde was prepared by heating a mixture of calcium propionate and formate and the butyric aldehyde by the oxidation of *n*-butyl alcohol with potassium dichromate and sulphuric acid. The methylene blue was Gruber's "Methylenblau med pur." The pyruvate solution was made by dissolving 1 g. of pyruvic acid in water, neutralising with *N* KHO and making to 100 cc.

Effect of the addition of pyruvate and acetaldehyde to yeast juice in presence and absence of free phosphate.

Exp. 1. 25 cc. Yeast-juice + 1 g. glucose. No toluene. $T = 25^{\circ}$.

	1% Pyruvate cc.	1% Acetaldehyde cc.	H ₂ O cc.		0.3M Na ₂ HPO ₄ cc.	H ₂ O cc.
1	5	0	0	and subsequently	5	0
2	5	0	0		0	5
3	0	5	0		5	0
4	0	5	0		0	5
5	0	0	5		5	0
6	0	0	5		0	5

Measurements were commenced at 2.20. The additions of phosphate and water were made at 3.20.

	Pyruvate 1 2		Aldehyde 3 4		Water 5 6	
cc. CO ₂ evolved before addition of phosphate in 55'	23.6	23.8	20.9	21.2	22.3	23.2
cc. CO ₂ evolved after addition of phosphate in successive periods of 5'						
	Na ₂ HPO ₄	H ₂ O	Na ₂ HPO ₄	H ₂ O	Na ₂ HPO ₄	H ₂ O
1	17.8	1.9	29.8	2	5.2	1
2	24.0	2	12.8	2	7.8	3
3)	6.7	4.6	7.4	3.7	28.1	4.2
4)						
5)	4.7	3.4	1.2	3.4	7.3	3.7
6)						
Total in 30'	53.2	11.9	51.2	11.1	48.4	11.9

It will be seen that the addition of pyruvate or aldehyde did not appreciably alter the normal rate of fermentation, as in the 55' preceding the addition of phosphate all six flasks gave approximately the same amounts of gas. Further,

the three flasks 2, 4 and 6 to which H_2O was subsequently added continued to ferment at equal rates, giving almost exactly equal amounts of gas in 30' (11.9, 11.1 and 11.9 cc.).

On the addition of phosphate, flask 6, containing no aldehyde or pyruvate, showed the normal behaviour, the rate gradually rising to a maximum which was attained in 10–15' and amounted to 15 per 5' (calculated by plotting the figures given in the table). In presence of aldehyde the rate rose to its maximum of 29.8 in the first 5', whilst in presence of pyruvate an intermediate result was obtained the evolution being 17.8 in the first 5' and 24 in the second.

Exp. 2. 25 cc. Yeast-juice + 1 g. glucose. No Toluene. $T = 25^\circ$.

	1% Pyruvate cc.	Water cc.		0.3M Na_2HPO_4 cc.	H_2O cc.
1	5	0	and subsequently	5	0
2	5	0		0	5
3	0	5		5	0
4	0	5		0	5

Measurements were commenced at 3.10 and the additions of phosphate and water were made at 3.40.

	Pyruvate		Water	
	1	2	3	4
cc. CO ₂ evolved before addition of phosphate between 3.10 and 3.30.				
	6.9	7.1	7.2	7
cc. CO ₂ evolved after addition of phosphate in successive periods of 5'.				
	Phosphate	Water	Phosphate	Water
1	3.4	0.3	0.3	0.5
2	5.6	0.5	1	0.7
3	4.4	0.4	0.8	0.4
4 } 5 }	10.2	0.8	1.6	0.8
Total in 25'	23.6	2	3.7	2.4
Total in 140'	49	9.7	31.2	9.2
Total in 17 hrs.	88.6	49.5	76.2	46.1

This is an example in which a quantity of phosphate largely in excess of the optimum was added. In the absence of added phosphate (Nos. 2 and 4) the fermentation was substantially the same with and without pyruvate. After addition of phosphate in the presence of pyruvate the rate rose much more rapidly than in its absence and in the latter case, probably owing to the continued action of excess of phosphate on the enzyme complex, the total was considerably less.

Effect of varying concentrations of acetaldehyde on the fermentation of glucose by zymine in presence and absence of phosphate.

Exp. 3. 4 g. Zymine + 2 g. Glucose in 20 cc. + 0.2 cc. Toluene. Acetaldehyde 1% solution (by weight).
T = 25.8°.

	1% Acetaldehyde	
	cc.	
1	0	} and subsequently 10 cc. 0.15M Na ₂ HPO ₄ to each.
2	1.2	
3	6	
4	12	

Incubated for 1 hour and the phosphate then added.

	1	2	3	4
	Acetaldehyde			
	0 cc.	1.2 cc.	6 cc.	12 cc.
cc. CO ₂ evolved before addition of phosphate in 45'.				
	34.4	33.6	20.7	3.4
cc. CO ₂ evolved after addition in successive periods of 5'.				
1	2.7	3.4	12.5	5.5
2	3.6	4.6	12.8	8.4
3	5.3	5	12.3	9.7
4	6.2	7.2	11.7	9.4
5	7.7	7.7	9.8	9.0
6	9.1	9.6	9.3	7.9
7	9.0	9.3	7.2	7.5
8	8.0	7.5	3.6	7.4
Total in 40'	51.6	54.4	79.1	64.8
Total in 110'	113.6	118.8	119.6	103.1

The characteristic effect is here well marked. In the control (No. 1) the maximum of 9.1 cc. is slowly reached in 30–35' and the rate then as gradually diminishes. 1.2 cc. of 1% aldehyde [1 in 1667] produce practically no effect, 6 cc. on the other hand [1 in 334] produce a very marked effect, the maximum is much higher (12.8 cc.) and is very rapidly attained (5–10'). The total evolved in 110' is however only about 5% greater than that of the control. A point of considerable interest is that both 6 cc. and 12 cc. of 1% aldehyde diminish the normal rate of fermentation although they both produce a considerable acceleration in the rate at which the maximum is attained after the addition of phosphate. This is especially marked in the case of the 12 cc. of aldehyde solution, which diminished the normal rate of fermentation to 1/10 of that of the control. This effect is probably due to a specific inhibition of the hexosephosphatase, thus diminishing the liberation of phosphate on which the normal rate depends.

Effect of formic, acetic, propionic and butyric aldehydes on the fermentation of glucose by yeast-juice in presence of phosphate.

Exp. 4. 25 cc. Yeast-juice + 1 g. Glucose + 0.2 cc. Toluene. T = 25°.

1	5 cc. Water	} and then 10 cc. 0.3M K ₂ HPO ₄ .
2	„ 0.78 % Formaldehyde	
3	„ 1 % Acetaldehyde	
4	„ 1.3 % Propionic Aldehyde	
5	„ 1.6 % Butyric Aldehyde	

Incubated for 50' before addition of phosphate.

	1	2	3	4	5
	Water	Formaldehyde	Acetaldehyde	Propionic Aldehyde	Butyric Aldehyde
cc. CO ₂ evolved in 30' before addition of phosphate					
	7.3	4.1	7.0	7.7	7.2
cc. CO ₂ evolved after addition of phosphate in successive periods of 5'.					
1 }	15.5	23.6	57.1	58.1	53.7
2 }					
3	12.5	22.4	16.4	16.6	16.1
4	15.3	20.7	2.7	2.7	2.8
5	18.4	7.0	1.6	1.5	1.7
6	11.9	1.4	1.2	1.6	1.4
7	2.5	1.5	1.8	1.6	1.4
	76.1	76.6	80.8	82.1	77.1

Exp. 5. As above, but 15 cc. of 0.3M K₂HPO₄ added. Incubated 25'.

	6	7	8	9	10
	Water	Formaldehyde	Acetaldehyde	Propionic Aldehyde	Butyric Aldehyde
cc. CO ₂ evolved after addition of phosphate in successive periods of 5'.					
1 } (10')	10.3	22.9	56.8 { 28.4	55.0 { 26	48.1 { 23
2 }			28.4	29	25.1
3	5.5	22.3	27.6	27.2	30.1
4	6.6	23.9	20.5	19.9	22.4
5	8.0	21.1	4.8	7.9	3.8
6	10.3	15.7	1.6	2.1	1.8
7	11.3	2.4	1.3	1.6	1.5
8	15.3	1.4	1.4	1.4	1.2
9	17.8				
10	13.9				
11 }					
12 }	4.5				

With this sample of juice very high maxima were attained, but as the first readings were made 10' after the addition of phosphate the exact maximum could not be directly observed. They were obtained by plotting the total fermentations and reading the evolutions per 5' from the curves, with the following approximate results.

Exp. 4.

	1	2	3	4	5
Maximum ...	18.4	22.4	34	35	27
Minutes after addition when attained ...	20-25	30-15	0-5	0-5	0-5

Exp. 5.

	6	7	8	9	10
Maximum ...	17.8	23.9	25.4	29	30.1
Minutes after addition when attained ...	40-45	15-20	0-5	5-10	30-15

The effect here is very marked and moreover in several cases the maximum is reached in the first 5' of fermentation. The results with the acetaldehyde in No. 8, Exp. 5, are plotted in Fig. 1.

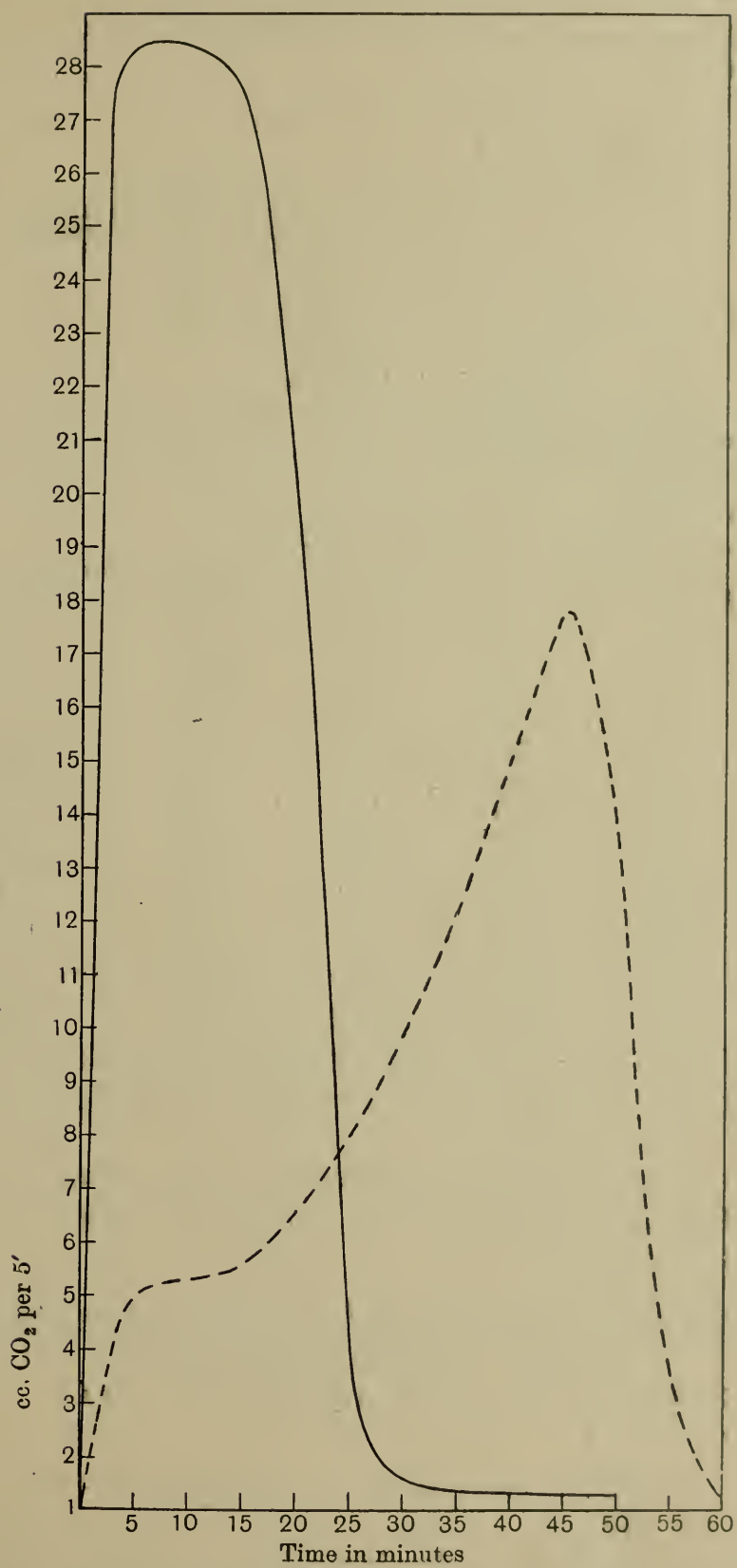


Fig. 1.

Effect of (a) Acetaldehyde, (b) Na Hexosephosphate on the fermentation of glucose by zymïn in presence of phosphate.

Exp. 6. 4 g. Zymïn + 2 g. Glucose in 20 cc. + 0.2 cc. Toluene. $T = 25.8^{\circ}$.

1% Acetaldehyde

1	0	} and subsequently	1	} 10 cc. 0.3M K_2HPO_4
2	1.2 cc.		2	
3	6 cc.		3	
4	0		4	10 cc. H_2O + 10 cc. 0.3M K_2HPO_4
5	0		5	Na Hexosephosphate from 0.2 g. Basalt + 0.52 g. K_2HPO_4 in 20 cc. (equivalent to 10 cc. 0.02M Hexosephosphate and 10 cc. 0.3M K_2HPO_4).

Incubated for 65' before the additions were made.

	Water 1	1.2 cc. Acetaldehyde 2	6 cc. Acetaldehyde 3	4	5
cc. CO_2 evolved before additions in 50'	39.2	33.8	28	38.9	38.4

Additions made at 1.05.

cc. CO_2 evolved after addition in successive periods of 5'.

	Phosphate	Phosphate	Phosphate	Phosphate and water	Phosphate + Hexose- phosphate
1	2.7	3.2	13.6	0.3	3.5
2	2.9	2.7	15.6	1.8	3.8
3	4.3	4.1	15.4	3.0	4.4
4	5.0	5.4	15.6	4.3	6.1
5	7.3	7.7	13.4	5.0	} 15.6
6	9.1	9.5	12.1	8.1	
7	10.5	10.7	11.1	9.2	8.8
8	11.4	12.3	10.0	10.3	8.8
9	11.0	10.4	8.0	10.3	8.6
10	10.9	10.8	6.4	9.2	8.0
11	9.6	9.1	3.6	8.9	} 14.6
12	8.4	8.1	3.4	8.6	
Total in 60'	93.1	94	128.2	79.0	82.4
Total in 120'	157	169.2	169.3	152.3	144.9

(a) The result with acetaldehyde confirms that obtained in Exp. 3, but the total in 2 hrs. is slightly larger in presence of acetaldehyde than in its absence.

(b) The addition of hexosephosphate has only a small effect in accelerating the attainment of the maximum. The total in 2 hours is slightly less than that in the control.

Effect of varying concentrations of methylene blue on the fermentation of glucose by zymïn in the presence of phosphate.

Exp. 7. 4 g. Zymïn + 2 g. Glucose in 20 cc. 0.2 cc. Toluene. $T = 25.5^{\circ}$.

1	Methylene Blue 0	} and subsequently 10 cc. 0.3M K_2HPO_4 to each.
2	" 0.1 g.	
3	" 0.2 g.	
4	" 0.3 g.	

The results of 1 and 2 are shown in the curves (Fig. 2) in which *A* and *B* represent the evolutions per 5' in absence and presence of methylene blue respectively.

In presence of 0.2 g. and 0.3 g. of methylene blue the course of the reaction was almost the same as with 0.1 g. but the maximum attained was in each case slightly lower (0.1 g., 13.2 cc.; 0.2 g., 12.5; 0.3 g., 12.7). The methylene blue in (2) became colourless in about 1 hr. and in (3) in about 2 hours.

The totals evolved in 2hr. 15 min. in the 4 flasks were almost identical, as will be seen from the following statement.

No.	M.B. g.	Max. attained (cc. in 5')	Total in 2 hrs. 15 min. cc.
1	0.0	13.2	181.2
2	0.1	13.2	184.5
3	0.2	12.5	176.8
4	0.3	12.7	178.9

When a still larger concentration of methylene blue is employed, the maximum is considerably lower than in its absence but is more quickly attained. The dye apparently partially inhibits the enzyme complex.

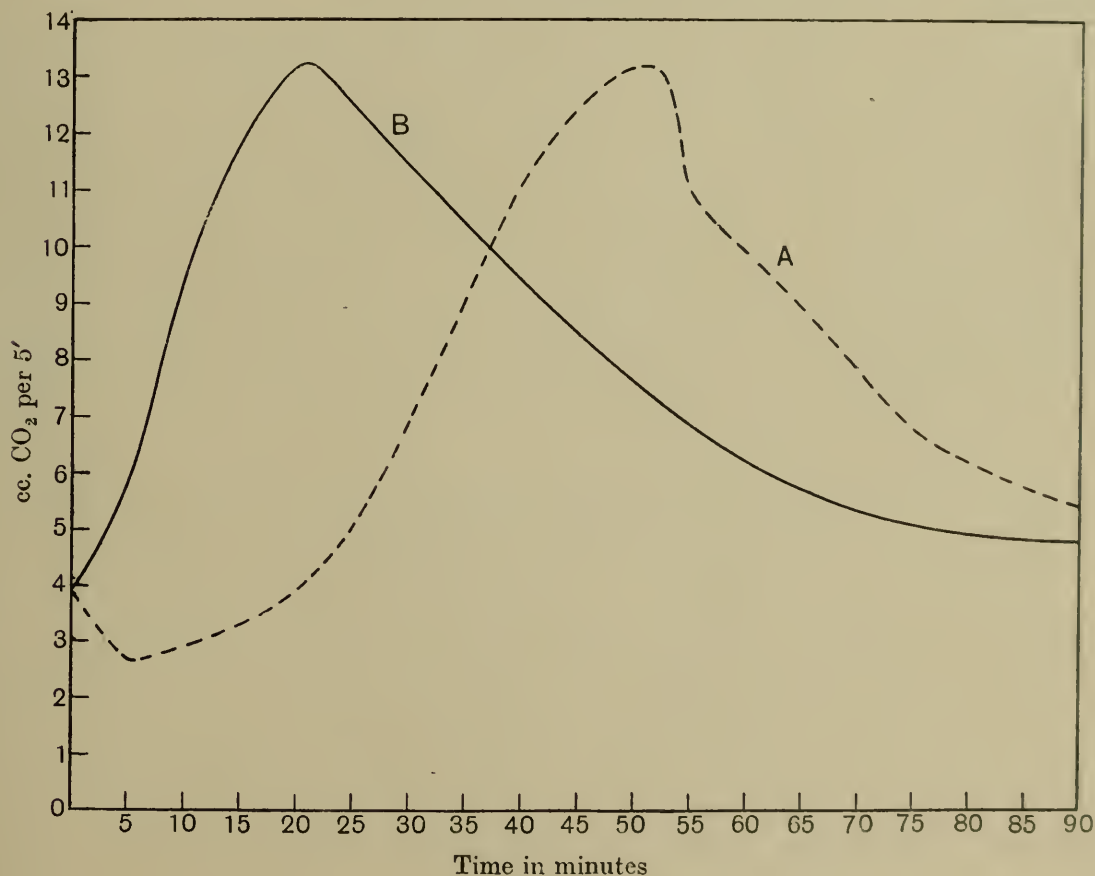


Fig. 2.

Exp. 8. 4 g. Zymin + 2 g. Glucose in 20 cc. + 0.2 cc. Toluene. $T = 26^\circ$.

1	Methylene Blue 0	} and subsequently	{ 6 cc. 0.3M K_2HPO_4
2	" 0.5 g.		
3	" 0		{ 10 cc. 0.3M K_2HPO_4
4	" 0.5 g.		

Result. After the addition of phosphate.

	Phosphate added 0.3M	M.B. g.	Max. attained	Time required to attain maximum	Total evolved in 1 hr. 35 min.
1	6	0	11	30-35'	107.0
2	6	0.5	8.8	0-5'	97.4
					in 1 hr. 55 min.
3	10	0	12.2	30-35'	147.1
4	10	0.5	10.6	5-10'	149.7

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XXI. THE EFFECT OF ACETALDEHYDE AND METHYLENE BLUE ON THE FERMENTATION OF GLUCOSE AND FRUCTOSE BY YEAST-JUICE AND ZYMIN IN PRESENCE OF PHOSPHATE AND ARSENATE.

BY ARTHUR HARDEN AND FRANCIS ROBERT HENLEY.

From the Biochemical Department, Lister Institute.

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It was previously shown by the authors [Harden and Henley, 1920, where the literature is quoted] that, in the fermentation of glucose in presence of phosphate by zymín or yeast-juice, acetaldehyde or methylene blue produced a remarkable acceleration in the early stages of the reaction and it was suggested that the function of these substances was to act as hydrogen acceptors and thus enable the reaction at once to attain a high rate. The experiments have now been extended to fructose, with the objects of ascertaining the maximum rates attainable with or without added acceptors and of comparing these with the corresponding rates yielded by glucose.

I. COMPARISON OF THE MAXIMUM RATES AND THE COURSE OF THE REACTION OBSERVED IN THE PRESENCE OF PHOSPHATE WITH GLUCOSE AND FRUCTOSE RESPECTIVELY IN THE PRESENCE AND ABSENCE OF ACETALDEHYDE.

A. *Zymín*. To 2 g. of zymín were added 10 cc. of a solution containing 1 g. of the sugar and, when acetaldehyde was used, 3 cc. of 1 % acetaldehyde; 0.2 cc. toluene was added in each case. This mixture was incubated at 25° until a constant rate of fermentation had been attained and varying volumes of 0.6 M K_2HPO_4 (previously saturated with CO_2) added. Readings were in many cases made every two minutes but the rates have usually been calculated for five minutes. With the smaller quantities of phosphate repeated additions were made when the rate began to slacken. This procedure was adopted because, especially in the case of glucose, the rate was depressed even by a small excess of phosphate. The final volumes therefore occasionally differ slightly, but this has very little effect on the maximum rate obtainable.

In Table I are given the maximum rates obtained with and without acetaldehyde, and the volumes of phosphate solution added.

Table I.

No.	Sugar	With 3 cc. 1 % acetaldehyde		Without acetaldehyde	
		Max. rate cc. per 5 min.	Additions	Max. rate cc. per 5 min.	Additions
1	Fructose	15	1 of 5 cc.	7.25	1 of 5 cc.
2	"	14	2 of 2 "	9	2 of 2 "
3	"	14	3 of 1 "	9.75	3 of 1 "
4	Glucose	9	2 of 2 "	7	1 of 2 "
5	"	7.5	2 of 1 "	5.5	1 of 1 "
6	"	9	1 of 2 "	—	—
7	"	8.25	1 of 2 " and 1 of 1 "	—	—
8	Cane-sugar	8.75	1 of 2 "	7.5	2 of 2 "

B. *Yeast-juice*. The experiments with yeast-juice were carried out in a similar manner to the above, 20 cc. of yeast-juice and 4 cc. of 1 % acetaldehyde being used, the total volume being 24.6 cc.; 0.2 cc. of toluene was added in each case. The results are embodied in Tables II and III, each of which refers to a different sample of yeast-juice.

Table II.

No.	Sugar	With 4 cc. 1 % acetaldehyde		Without acetaldehyde	
		Max. rate cc. per 5 min.	Additions	Max. rate cc. per 5 min.	Additions
9	Glucose	16	1 of 1 cc.	10.75	3 of 1 cc.
10	"	18.5	1 of 1 " and 1 of 2 "	11.75	2 of 2 "
11	"	21	1 of 5 "	—	—
12	Fructose	29.6	1 of 5 "	24.5	1 of 5 "
13	"	36	1 of 10 "	33	1 of 10 "

Table III.

14	Fructose	17.25	1 of 5 cc.	—	—
15	"	18.25	4 of 2 "	—	—
16	Cane-sugar	8.25	1 of 5 "	5.5	1 of 5 "
17	"	11.25	2 of 2 "	10	1 of 2 "

Owing to the fact that repeated additions of phosphate were made in most of these cases, the course of the reaction was irregular. Fig. 1 however shows the characteristic effect exerted by acetaldehyde on the fermentation of fructose in presence of phosphate (experiment B 13 above). In this case the rates in cc. per 2 min. are plotted against the time, and it is seen that in the presence of aldehyde the rate rises much more rapidly, but only to a slightly higher maximum.

It will be seen from the above that the maximum rate obtainable from glucose under the optimum conditions of phosphate and aldehyde falls far short of that observed with fructose. For the sample of zymine employed for experiments A 1–8 the maximum rates in presence of aldehyde were 15

for fructose and 9 for glucose, a ratio $F/G = 15/9 = 1.67$. In the case of the yeast-juice used in experiments B 9-13 the ratio $F/G = 36/21 = 1.7$, almost identical with that for zymin.

It appears to follow from this, since all these rates were determined in presence of an excess of acceptor, that the difference in the behaviour of fructose and glucose although partly attributable to a difference in the rate of production or of reduction of acceptor, cannot be wholly accounted for in this way, but must extend to some other stage in the reaction.

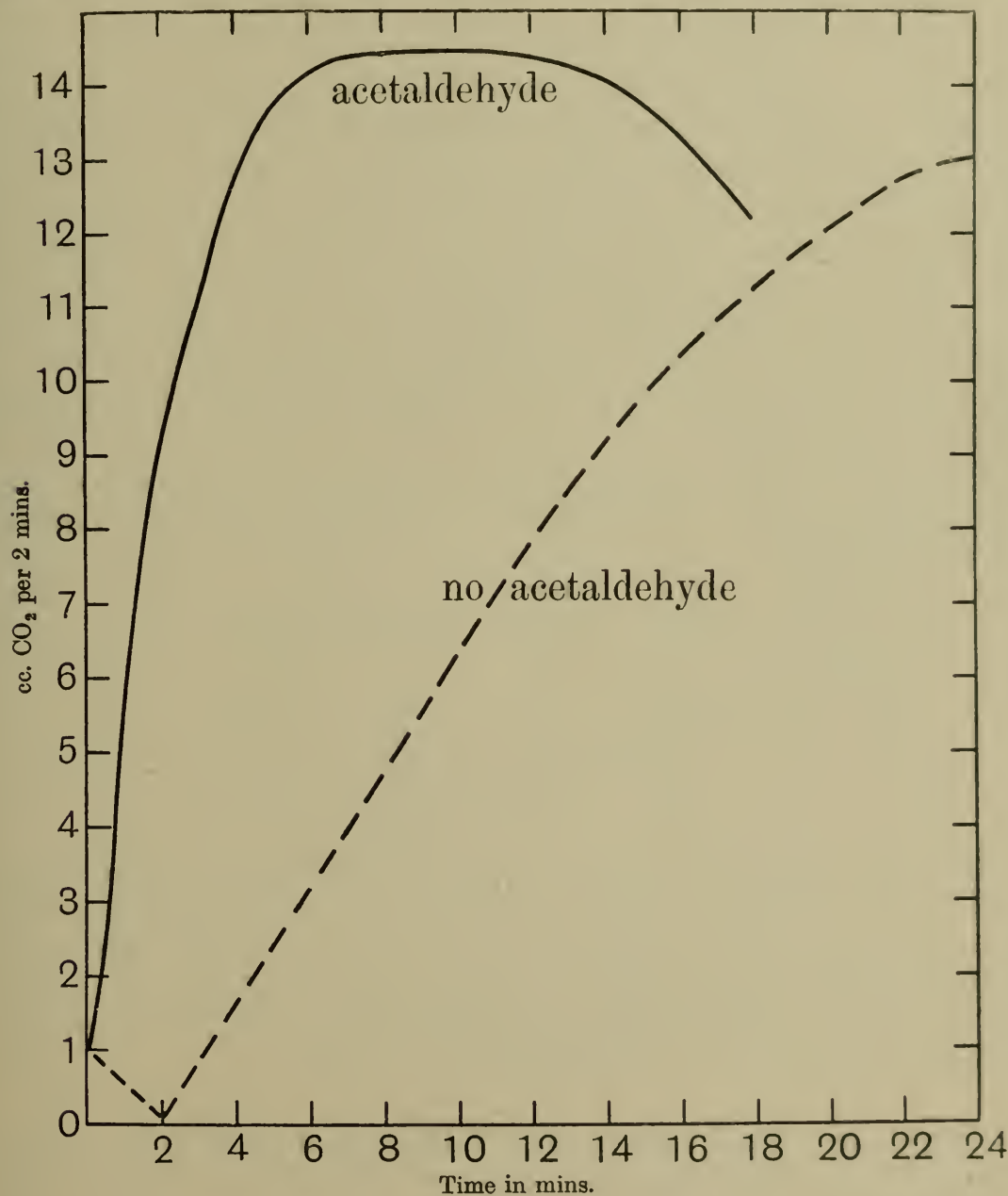


Fig. 1.

II. RELATIVE EFFICIENCY OF ACETALDEHYDE AND FRUCTOSE AS AGENTS OF "INDUCTION."

It was suggested in the previous paper [Harden and Henley, 1920] that the inductive effect of fructose [Harden and Young, 1909] on a mixture of

glucose and phosphate was due to the more ready production of acceptor from fructose than from glucose. This might occur in either or both of two ways: firstly, by the more rapid production from fructose of a substance capable of acting as an acceptor in the preliminary stage of the reaction (possibly identical with the precursor of glycerol and possibly a different substance from that produced from glucose) or by the more rapid reduction of such a preliminary acceptor under the prevailing conditions; secondly by the more rapid fermentation of the fructose, yielding as intermediate product acetaldehyde (which may be termed the normal acceptor) which would then be available to complete the cycle of fermentation of further molecules either of fructose or of glucose, even in the presence of a considerable excess of phosphate. Under these circumstances it became of interest to ascertain the relative efficiencies of fructose and acetaldehyde in this respect. Experiments were therefore made with yeast-juice and glucose in presence of excess of phosphate by adding varying amounts of acetaldehyde and fructose and comparing their effects, with the result that a concentration of 1/42,200 of acetaldehyde gave almost exactly the same reaction curve as 1/211 of fructose (see Fig. 2), this latter being equivalent to 10 % of the glucose present. In other words acetaldehyde is 50 times as effective as fructose, when molecular quantities are compared.

Experiment 18. Six quantities of 25 cc. yeast-juice + 2 g. glucose + 10 cc. 0.6 M K_2HPO_4 + 0.2 cc. toluene were incubated for ten minutes at 25° and then were added:

No.	10 % glucose cc.	10 % fructose cc.	1 % acetaldehyde cc.	Water cc.
1	2	0	0	4
2	2	0	0.1	3.9
3	2	0	0.5	3.5
4	2	0	1.0	3
5	2	0	1.5	2.5
6	0	2	0	4

The course of the fermentations in the various flasks is shown by the curves of Fig. 2, rates in cc. per five minutes being plotted against time. The curve for (4) was almost coincident with that for (5), whilst that for (3) was intermediate between those for (4) and (2). It will be seen that the addition of 0.1 cc. of acetaldehyde solution (2) produced almost exactly the same effect as that of 2 cc. of the fructose solution (6).

III. EFFECT OF ACETALDEHYDE ON THE FERMENTATION OF GLUCOSE AND FRUCTOSE BY YEAST-JUICE AND ZYMIN IN PRESENCE OF ARSENATE AND PHOSPHATE.

It has previously been shown [Harden and Young, 1911] that arsenate greatly accelerates the action of the hexosephosphatase of yeast-juice and zymin so that the fermentation of glucose and fructose proceeds much more rapidly in the presence of a suitable concentration of arsenate than in its

absence. Excess of arsenate, however, produces inhibition. It therefore became of interest to ascertain whether the arsenate, in addition to its effect on the hexosephosphatase, exerted any modifying influence on any other stage of the fermentation and in particular whether it in any way affected the accelerating action of acetaldehyde, a possibility which has not previously been considered.

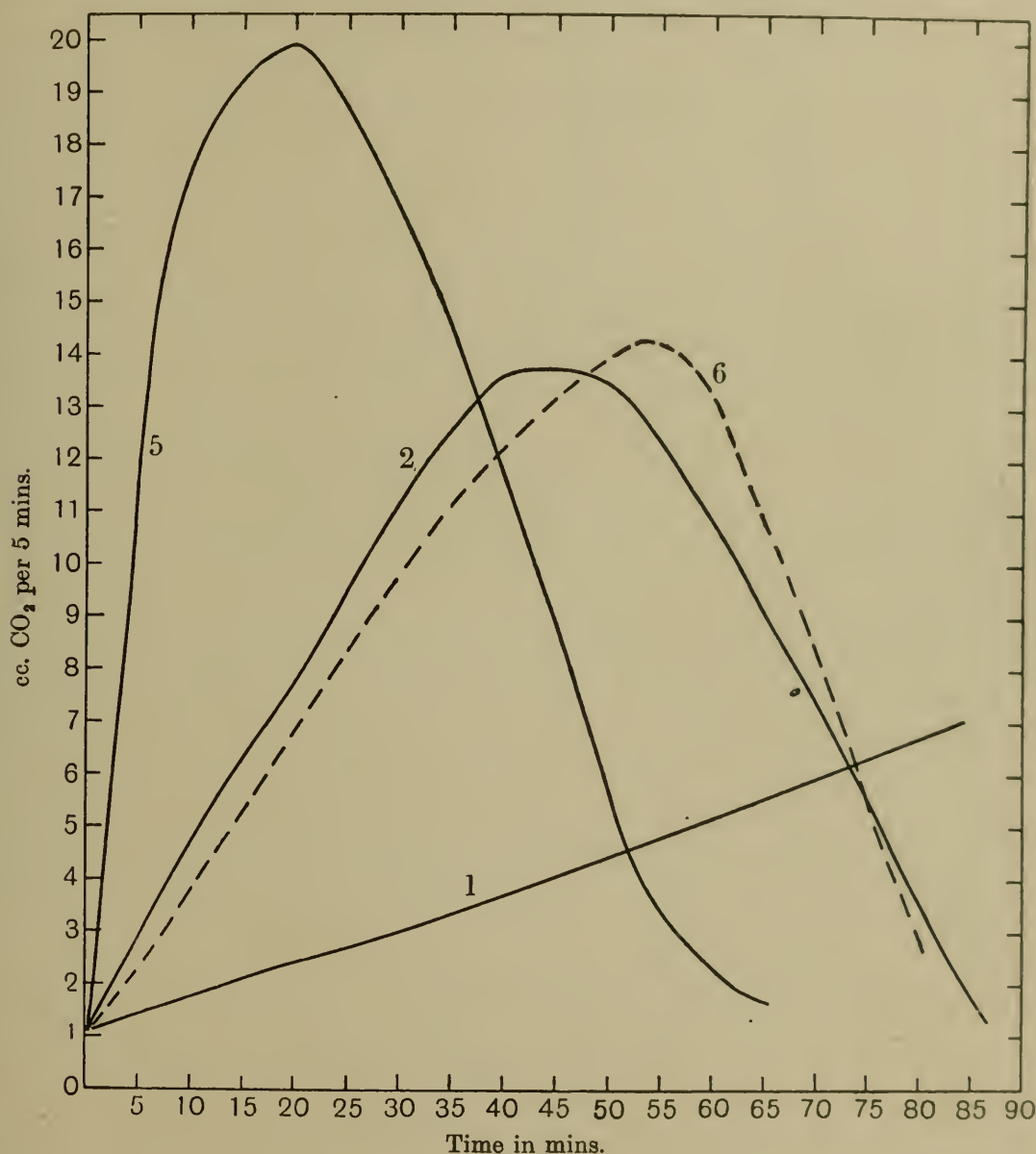


Fig. 2.

C. Yeast-juice.

Experiment 19. Six flasks were made up as follows:

No.	Yeast-juice cc.	Sugar	Toluene cc.	Water cc.	1 % acetaldehyde		
					cc.	0.3 M Na ₂ HAsO ₄ cc.	0.6 M K ₂ HPO ₄ cc.
1	20	2 g. fructose	0.2	15	0	0	5
2	20		0.2	10	5	0	5
3	20		0.2	14	0	1	5
4	20		0.2	9	5	1	5
5	20		0.2	10	0	5	5
6	20		0.2	5	5	5	5

The sodium arsenate and potassium phosphate were added to the mixture after it had been incubated for 30 mins. at 25°. The course of the fermentation in the different flasks is shown by the curves of Fig. 3.

A similar experiment was carried out with glucose, a different sample of yeast-juice being employed, which was capable of producing fermentation in presence of an exceptionally high concentration of phosphate.

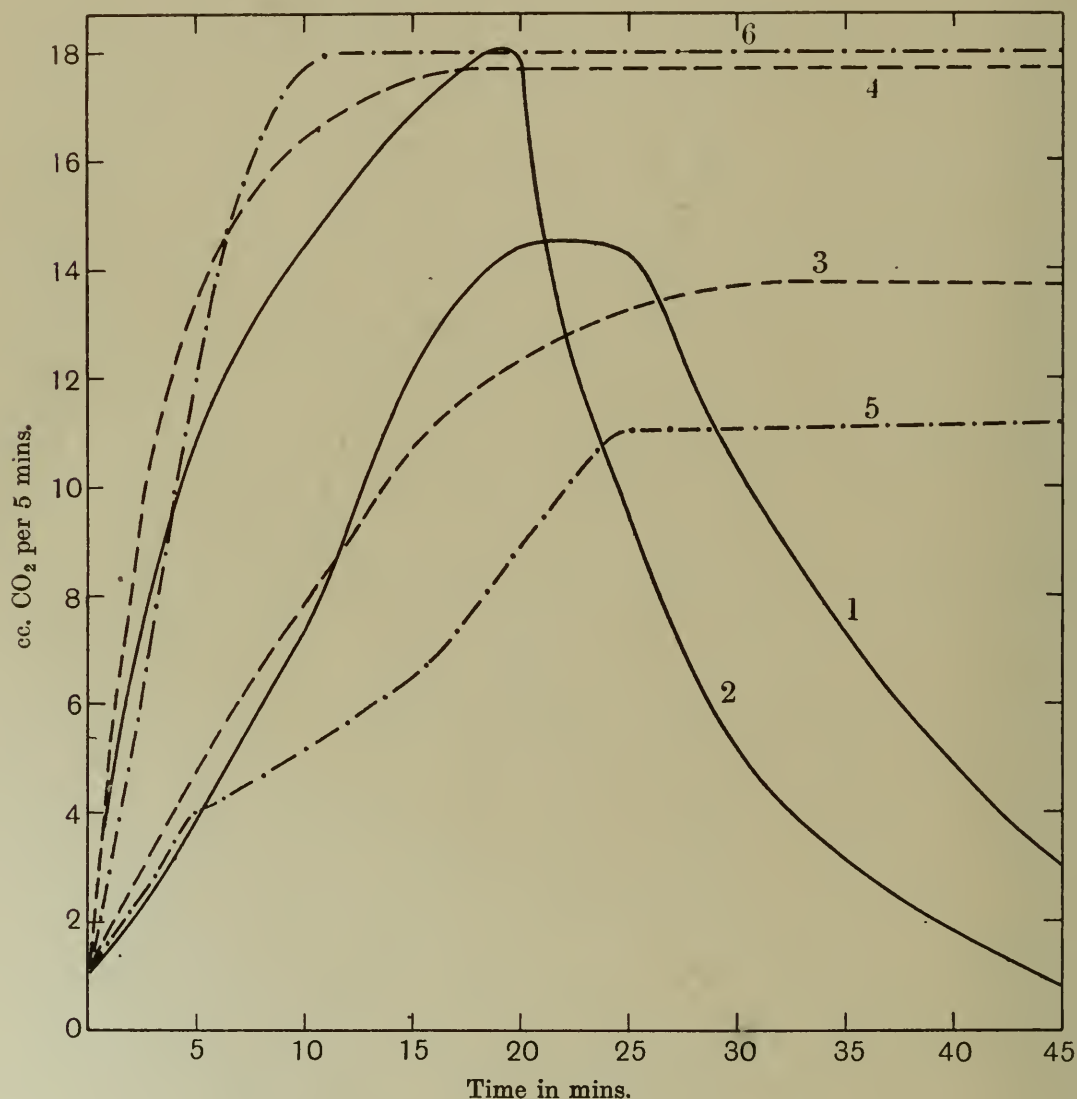


Fig. 3.

Experiment 20. Six flasks were made up, each containing 20 cc. yeast-juice + 2 g. glucose + 0.2 cc. toluene together with:

No.	Water cc.	1 % acetaldehyde cc.	0.3 M Na ₂ HAsO ₄ cc.	0.6 M K ₂ HPO ₄ cc.
7	12.5	0	0	7.5
8	7.5	5	0	7.5
9	11.5	0	1	7.5
10	6.5	5	1	7.5
11	7.5	0	5	7.5
12	2.5	5	5	7.5

The sodium arsenate and potassium phosphate were added to the mixture after it had been incubated for 30 mins. at 25°.

The course of the fermentation was very similar to that observed in experiment 19 with fructose, as is indicated by the following observations:

No.	Arsenate cc.	Acetaldehyde cc.	Maximum rate cc. per 5 min.	Time (min.) re- quired to attain max.
7	0	0	18	60
8	0	5	33.6	10
9	1	0	16.8	85
10	1	5	35.9	10
11	5	0	(7.1)	90
12	5	5	22.7	10

The small increase of rate in 10 as compared with 8 is probably due to the fact that in 8 the optimum concentration of phosphate was not present, whereas in 10, owing to the action of the arsenate, optimum conditions were maintained.

Two interesting points emerge from these experiments:

1. In presence of excess of phosphate, arsenate does not remove the inhibition, but somewhat increases it (Curves 1, 3, 5, Fig. 3 and experiment 20, Nos. 7, 9, 11).

2. The presence of arsenate does not affect the accelerating action of acetaldehyde in presence of phosphate (Curves 3, 4, 5 and 6, Fig. 3 and experiment 20, Nos. 8, 10).

D. Zymin.

Experiment 21. Twelve flasks were made up each containing 2 g. zymin with the following additions; 0.2 cc. of toluene was added in each case:

No.	Sugar	Water cc.	1 % acetaldehyde cc.	Added subsequently			Water cc.
				0.6 M K_2HPO_4 cc.	0.3 M Na_2HAsO_4 cc.		
1	1 g. glucose	9.4	0	2	0		1.2
2	"	6.4	3	2	0		1.2
3	"	9.4	0	2	0.1		1.1
4	"	6.4	3	2	0.1		1.1
5	"	9.4	0	2	0.2		1
6	"	6.4	3	2	0.2		1
7	1 g. fructose	9.4	0	3	0		0.2
8	"	6.4	3	3	0		0.2
9	"	9.4	0	3	0.1		0.1
10	"	6.4	3	3	0.1		0.1
11	"	9.4	0	3	0.2		0.2
12	"	6.4	3	3	0.2		0.2

The course of the reaction is indicated by the following observations:

No.	Sugar	Arsenate cc.	Acetaldehyde cc.	Maximum rate cc. per 5 min.	Time (min.) re- quired to attain maximum
1	Glucose	0	0	5.9	40
2	"	0	3	6.2	10
3	"	0.1	0	6.8 (average)	45
4	"	0.1	3	9.6 "	10
5	"	0.2	0	6.9 "	45
6	"	0.2	3	9.9 "	10
7	Fructose	0	0	9.8	35
8	"	0	3	12.1	10
9	"	0.1	0	10.1 (average)	35
10	"	0.1	3	14.1 "	10
11	"	0.2	0	9.9 "	40
12	"	0.2	3	14.4 "	10

In its main features the zymin behaves in a similar manner to yeast-juice. Owing to the small concentration of arsenate employed, the inhibiting effect of the arsenate, which was marked in the case of yeast-juice, is here barely perceptible.

Another point of difference is that the maximum rate, especially with glucose, in presence of arsenate is considerably greater than in its absence, the two rates in question being 6.2 and 9.9 or a ratio of 1.6, whereas with yeast-juice and glucose the ratio is $33.6/35.9 = 1.07$. The difference is much less with fructose, the corresponding ratios being 1.2 for zymin and 1.0 for yeast-juice. In order to ascertain whether a real difference of this kind exists between yeast-juice and zymin, experiments were made in which the maximum rate in presence of the optimum concentration of phosphate in presence of acetaldehyde was determined, and arsenate was then added to the same mixture. With glucose this produced, as in the previous experiment, a marked rise of rate, whereas with fructose only a small and insignificant rise was observed. This shows that the arsenate exerts a definite accelerating effect on the fermentation of glucose by zymin apart from its action on the hexose-phosphatase. The exact significance of the phenomenon has not yet been ascertained.

Experiment 22. 2 g. zymin + 1 g. of the sugar (glucose or fructose) + 3 cc. 1 % acetaldehyde + 6.44 water + 0.2 cc. toluene were incubated at 25° until the rate was constant. Successive small additions of phosphate were made, as in experiments 1-8, until the maximum rate obtainable had been observed. 0.2 cc. of 0.3 M Na_2HAsO_4 was then added and the average rate attained was noted.

Sugar	Maximum (cc. per 5 min.)	
	In absence of arsenate	In presence of arsenate
Glucose	6.0	8.5
Fructose	13.0	13.5

IV. EFFECT OF METHYLENE BLUE.

It was shown in the previous communication [Harden and Henley, 1920] that methylene blue behaved in a similar manner to acetaldehyde with respect to the fermentation of glucose by zymin in presence of phosphate. These experiments have now been extended to the fermentation of both glucose and fructose in the presence of phosphate, with the result that the methylene blue has been found to behave in a similar manner in all these cases.

The experiments were carried out exactly as in the case of acetaldehyde, but the methylene blue was added in the solid form. No attempt was made to ascertain the optimum conditions, so that the rates observed are not necessarily the maximum obtainable.

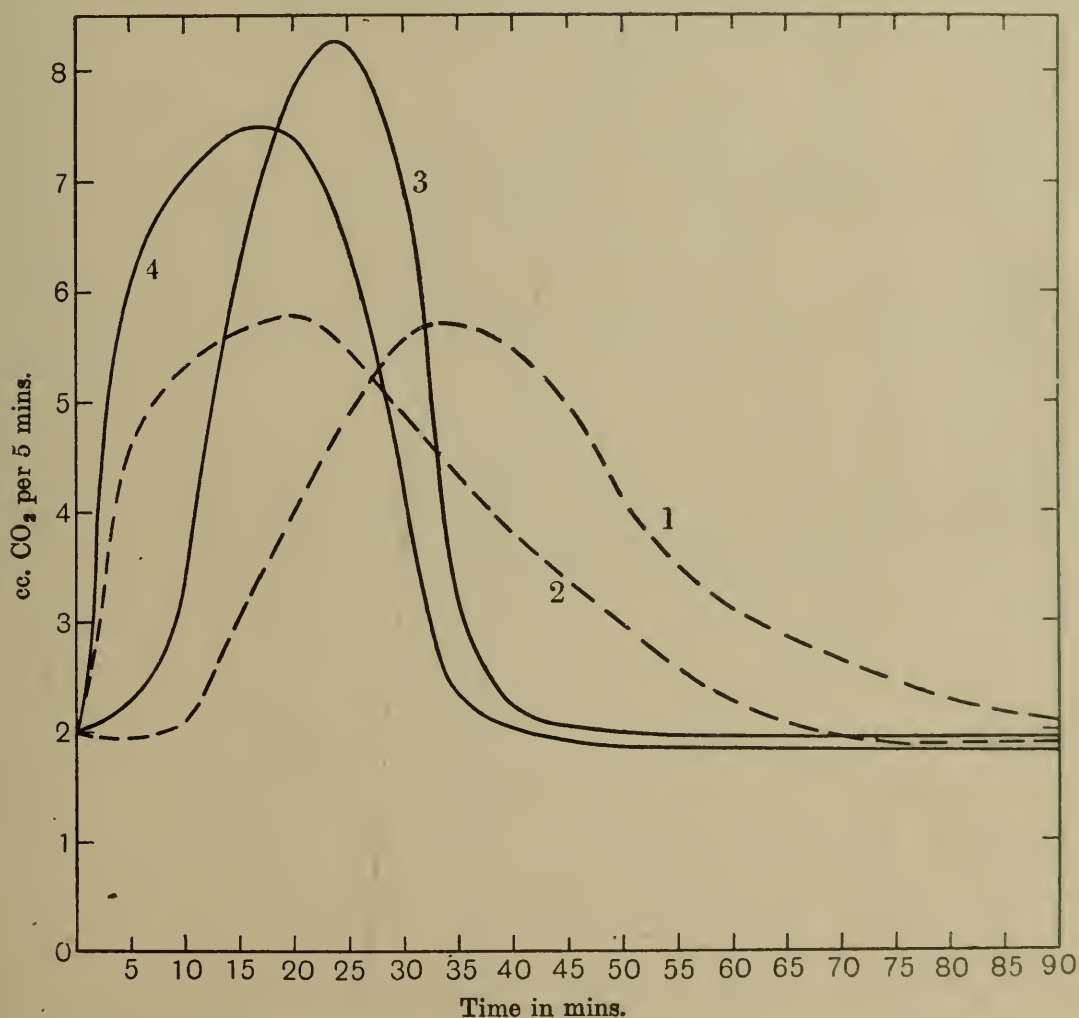


Fig. 4.

E. Zymin and glucose and fructose.

Experiment 23. Four flasks were made up containing each 2 g. zymin + 1 g. of sugar + 9.4 cc. water + 0.2 cc. toluene. Nos. 1 and 2 contained glucose, Nos. 3 and 4 fructose. 0.1 g. methylene blue was added to Nos. 2 and 4. These were incubated for 35 mins. at 25° and 2 cc. 0.6 M K_2HPO_4 were then added to each. The course of the reaction is seen from the curves (Fig. 4).

F. *Yeast-juice and glucose and fructose.*

Experiment 24. Four flasks were made up containing

- | | | | | |
|----|--------------------|-----------------|-------------------|-------------------------|
| 1. | 25 cc. yeast-juice | + 2 g. glucose | + 0.2 cc. toluene | |
| 2. | " " | + " | + " | + 0.2 g. methylene blue |
| 3. | " " | + 2 g. fructose | + " | |
| 4. | " " | + " | + " | + " " |

These were incubated for 15 mins. at 25° and 15 cc. 0.6 M K_2HPO_4 were then added. The course of the reaction is shown in the accompanying curves (Fig. 5).

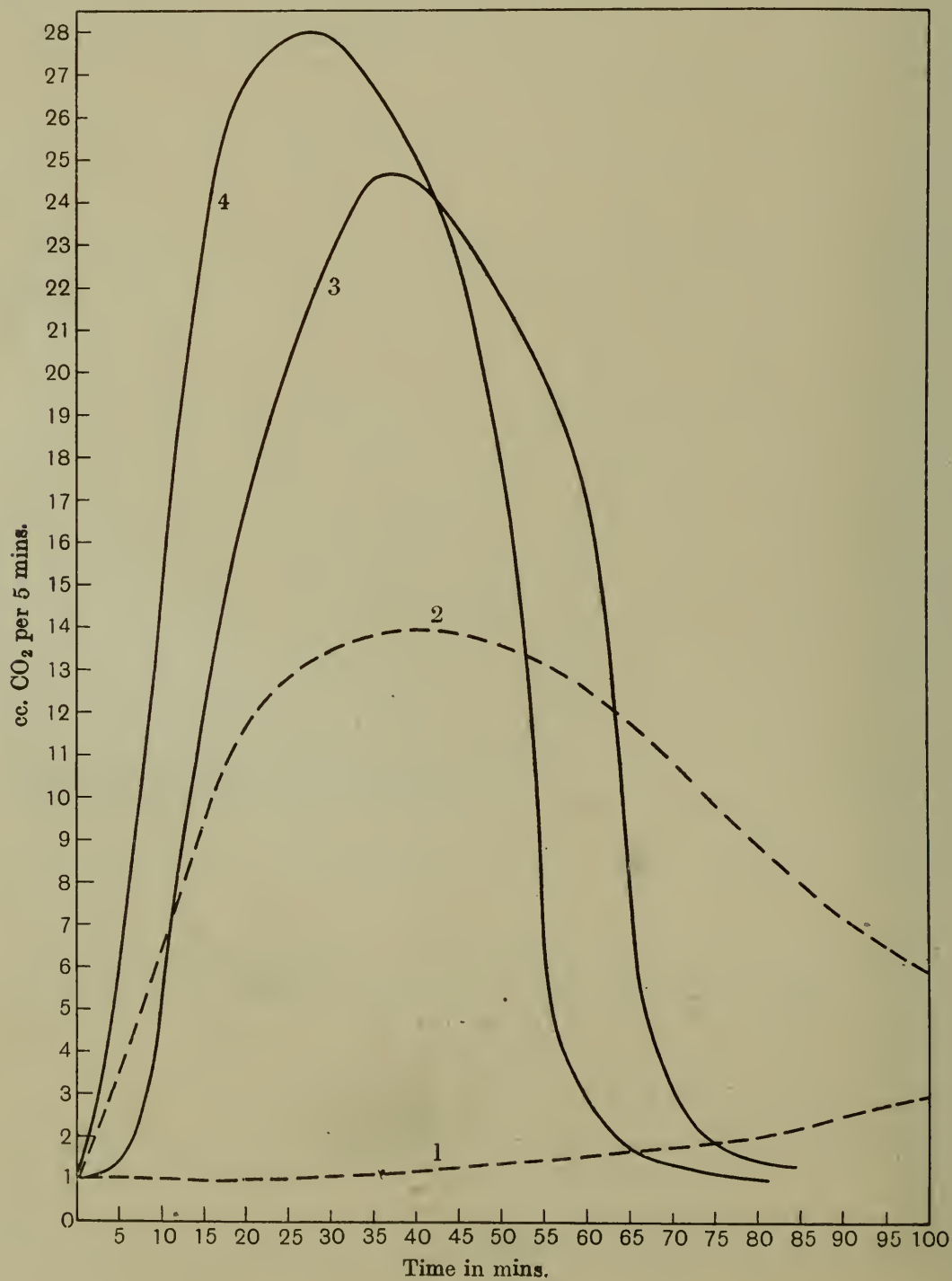


Fig. 5.

In every case the addition of the methylene blue caused a marked acceleration in the rate of attainment of the maximum. The concentration of the phosphate was so high as greatly to inhibit the fermentation of the glucose in absence of methylene blue (curve 1).

SUMMARY.

1. Acetaldehyde diminishes the time required by a mixture of fructose and phosphate to attain its maximum rate of fermentation in presence of yeast-juice or zymin, but does not substantially increase the maximum rate obtainable.

2. In presence of acetaldehyde fructose is more rapidly fermented than glucose in presence of phosphate both by yeast-juice and zymin.

3. Acetaldehyde is about 50 times as effective as fructose (when molecular quantities are compared) in "inducing" fermentation in a mixture of glucose with excess of phosphate.

4. Arsenate does not affect the accelerating action of acetaldehyde in presence of phosphate in the fermentation of glucose and fructose by yeast-juice but considerably increases the rate attained with glucose, and to a less degree that attained with fructose, when zymin is used.

5. Methylene blue produces an effect similar to that of acetaldehyde in the fermentation of both fructose and glucose by zymin and yeast-juice in presence of phosphate.

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XXXIV. THE SALT EFFECT IN ALCOHOLIC FERMENTATION.

By ARTHUR HARDEN AND FRANCIS ROBERT HENLEY.

From the Biochemical Department, Lister Institute.

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WHEN the necessary hydrogen acceptor (in the form of acetaldehyde) is supplied to a mixture of yeast juice and sugar containing the appropriate concentration of phosphate, the maximum rate of fermentation is attained with practically no delay [Harden and Henley, 1920]. If, however, the acetaldehyde be omitted, the other conditions remaining unchanged, there will be a considerable delay in attaining the maximum rate of fermentation. With increasing concentration of phosphate, the period of delay is lengthened, as the maximum rate of fermentation is in general not attained until the concentration of phosphate has been reduced approximately to the optimum by the formation of hexosephosphate (see Figs. 1 and 2). If the concentration of phosphate be raised still more a point is reached at which the rate of fermentation never reaches the maximum. In presence of added acceptor (acetaldehyde) the maximum rate of fermentation attainable and optimum concentration of phosphate are higher than in its absence [Harden and Henley, 1920].

It appears therefore that the enzymes concerned are extremely sensitive to even a slightly excessive concentration of phosphate.

Meyerhof [1918] has shown that NaCl and other salts exercise a similar depressing effect both on the rate of attainment of the maximum and on the maximum rate attained and he therefore considers that phosphate, in addition to its specific function, exerts a general effect on the process which is shared by other salts. The following experiments were undertaken with the object of comparing the relative effects of different salts and of ascertaining whether the effect is a general one or is specific to any particular stage of the fermentation process.

It was found in the first place, in full confirmation of Meyerhof's results, that the effect of other salts is in general similar to that of excess of phosphate. Using equimolecular solutions the chlorides of Na and K have about the same effect, which is, however, less than that of the corresponding sulphates.

As regards the effect in different stages of the process, direct experiment showed that chlorides and sulphates, in the concentrations employed in these experiments, had no depressing effect on the rate of decomposition of pyruvic acid, whilst phosphates had a slightly stimulating effect, probably due to the

diminution of $[H']$ in their presence. It was next established that the characteristic salt effect was not removed or greatly modified by the addition of acetaldehyde. Finally it was found that the rate of action of the hexose-phosphatase was diminished by the salts employed.

As regards the effect of excess of phosphate it was found that this differs from that of sulphates and chlorides by being relatively greater in the absence of acetaldehyde and less in its presence.

Experimental.

I. EFFECT OF $NaCl$, Na_2SO_4 , KCl AND K_2SO_4 ON THE FERMENTATION OF FRUCTOSE AND GLUCOSE BY ZYMIN.

Expt. 1. The fermenting mixtures had a concentration $0.1 M$ of K_2HPO_4 and $0.25 M$ of the experimental salt. The maxima attained and the times required for their attainment were as follows:

	Maximum rate cc. per 5 mins.	Time mins.
Control	7.7	35
$NaCl$	5.2	48
Na_2SO_4	4.3	57
KCl	5.2	45
K_2SO_4	4.4	52

The results with glucose and fructose were similar. Meyerhof's observations are thus confirmed and it is further to be noticed that the chlorides of Na and K have about the same effect, which is less than that produced by the corresponding sulphates. The greater effect of these latter salts indicates that it is probably the anion of the salt which is efficacious.

II. EFFECT OF SALTS ON PARTICULAR STAGES OF THE FERMENTATION PROCESS.

A. *Effect of K_2HPO_4 , KCl and K_2SO_4 on the action of carboxylase.*

In order to obviate the difficulties introduced by autofermentation, washed zymin was used in presence of a pyruvate and boric acid [see Harden, 1913]. The pyruvate solution was made by neutralising 2.42 g. of pyruvic acid with KHO and making to 25 cc.

Expt. 2. Five flasks were made up each containing 10 cc. of washed zymin suspension (2 g. zymin), and 0.2 cc. toluene with the following additions, the total volume being 15 cc.

No.	Glucose	Pyruvate	Boric acid	$1.2 M K_2HPO_4$	$1.2 M KCl$	Water
1	0.5 g.	0 cc.	0 g.	0.75 cc.	0.5 cc.	13.5 cc.
2	0	6	1.2	0	0	9
3	0	6	1.2	9	0	0
4	0	6	1.2	0	4	5
5	0	6	1.2	0	9	0

After incubation for 10 minutes at 25° , readings of the gas evolution were taken at frequent intervals, with the following results. The absence of fermentation in No. 1 shows that the washing had been efficacious.

No.	Total gas evolved		
	1 hour	2 hours	4 hours
1	0.1 cc.	0.1 cc.	0.1 cc.
2	15.3	18.9	25.6
3	19.0	26.8	33.7
4	13.4	18.6	23.0
5	13.7	19.0	23.2

By comparing the results of Nos. 2, 4 and 5, it is seen that the KCl has had practically no effect, though present in higher concentration than in the experiment previously described (Expt. 1). A similar result was obtained on another occasion with K_2SO_4 .

The K_2HPO_4 has had a slight beneficial effect as the total gas produced in No. 3 is greater than that in No. 2. This effect may perhaps be due to a lowering of the $[H^+]$ by the K_2HPO_4 .

B. *Effect of K_2SO_4 and of acetaldehyde on the rate of hydrolysis of hexose-phosphate by hexosephosphatase.*

In presence of excess of sugar the rate of fermentation produced by either yeast juice or zymin is conditioned by the concentration of free phosphate. As soon as any free phosphate present in the juice has been converted into hexosephosphate the rate of renewal of the supply depends mainly on the rate of action of the hexosephosphatase present. In these circumstances the rate of fermentation is a measure of the rate of action of the hexosephosphatase [see Harden, 1914, p. 119]. By adding a salt solution to a fermenting mixture in which these conditions have been attained, it is possible to determine whether the added salt has any effect on the hexosephosphatase rate.

A study of the effect of phosphate on the rate of action of hexosephosphatase cannot be made in this way because it enters into the reaction and the active masses of the reacting substances are thereby changed.

The effect of K_2SO_4 was tried both in the absence and presence of acetaldehyde for the sake of comparison with later experiments.

Expt. 3. Four flasks were made up each containing 2 g. zymin + 2 g. glucose + 0.2 cc. toluene and the following additions were made:

No.	1 % acetaldehyde	Water	0.4 M K_2SO_4	
1	0 cc.	20.9 cc.	0 cc.	
2	3	17.9	0	
3	0	13.4	7.5	} Added after the steady rate had been attained.
4	3	10.4	7.5	

After a steady rate had been attained, readings were made every 5 minutes, with the following results:

No.	1	Average rate over 125 minutes 0.71 cc. CO_2 per 5 mins.			
2	"	"	"	0.71	"
3	"	"	80	0.45	"
4	"	"	"	0.59	"

The added K_2SO_4 has caused a marked reduction in the rate but this is not so great in the presence of acetaldehyde.

C. *Salt effect in presence and absence of added acceptor (acetaldehyde).**Comparison of effects produced by K_2SO_4 and K_2HPO_4 .*

Expt. 4. Six flasks were made up each containing 20 cc. of yeast-juice + 2 g. fructose + 0.2 cc. toluene, and the following additions were made:

No.	1 % acetaldehyde	Water		0.4 M K_2SO_4	0.6 M K_2HPO_4	Water
1	0 cc.	5 cc.	} And after incubation at 25° for 25 mins.	0 cc.	10 cc.	7.5 cc.
2	5	0		0	10	7.5
3	0	5		7.5	10	0
4	5	0		7.5	10	0
5	0	5		0	15	2.5
6	5	0		0	15	2.5

Readings of the gas evolution were taken at intervals of 5 minutes.

As the concentration of phosphate in the fermenting liquid is progressively changing, whereas the concentration of K_2SO_4 presumably remains unchanged, it is useless to make a direct comparison of the rates of fermentation observed in the several experiments at equal intervals of time measured from the moment at which the various additions were made. Since a simple relation exists between the K_2HPO_4 added and the CO_2 evolved in excess of that which would have been produced had no K_2HPO_4 been added, it is possible to calculate from the total gas evolution, measured from the time at which additions were made, the amount of free phosphate remaining at any moment during the course of the experiment. When this calculation has been made at a series of intervals during each experiment, comparison of the various experiments can be made at equal concentrations of free phosphate. In making the calculations it is assumed that 1 cc. of 0.6 M K_2HPO_4 is equivalent to 14.8 cc. of CO_2 under the conditions of the experiment, and that 0.9 cc. CO_2 must be allowed as the normal rate of fermentation in absence of added phosphate. The latter assumption is not strictly accurate for all the experiments, since the normal rate of fermentation, *i.e.* the hexosephosphatase rate, is affected by the addition of phosphate or sulphate as shown in section B above. The errors introduced from this cause are probably not large, and are to some extent unavoidable, as it is very difficult to estimate the hexosephosphatase rate exactly after the addition of large amounts of phosphate. The curves shown in Figs. 1 and 2 have been prepared in this way by calculation from the observed gas evolution in experiment 4 (Nos. 1-6). In the cases (Nos. 5 and 6) in which the effect of 5 cc. of extra phosphate is to be determined, it is necessary to assume the available free phosphate as equivalent to only 10 cc. of the total of 15 cc. which are actually present, and only to utilise the observations made while the extra 5 cc. of phosphate remained intact. Hence the comparison can only be carried as far as the point at which the concentration of phosphate in the experiments with 10 cc. of phosphate alone, or in presence of K_2SO_4 (Nos. 1, 2, 3, 4) is such as to produce the maximum rate. After this point the rate in these experiments falls off for lack of phosphate, whereas in the experiments with the extra 5 cc. of phosphate (Nos. 5 and 6) this does not occur so soon, and the rate maintains a higher level at the expense of some of the extra phosphate.

In order to compare the depressing effects of the added salts the ratio of the rates of fermentation, at points on the curves where the available phosphate concentration is equal, are employed. These ratios may legitimately be calculated at any points of equal phosphate concentration. But it should be noted that the results at the beginning and end of the experiments are not so trustworthy as the others. In the former case, because the rates are changing rapidly, in the latter because with diminishing phosphate and rate of fermentation the relative errors of calculation are greatly increased.

The results of the observations in absence of added acetaldehyde are shown in Fig. 1, and in presence of acetaldehyde in Fig. 2.

When the curves 1 and 3 (K_2SO_4 in absence of acetaldehyde) and 2 and 4 (K_2SO_4 in presence of acetaldehyde) are compared it is found that the ratios are fairly constant over a considerable range of free phosphate concentration, the values being given in the following table.

Depressing effect of K_2SO_4 .

Available phosphate in cc. of CO_2	Without acetaldehyde		With acetaldehyde	
	Ratio	$\frac{\text{Rate from 3}}{\text{Rate from 1}}$	Ratio	$\frac{\text{Rate from 4}}{\text{Rate from 2}}$
135		0.78		0.77
130		0.78		0.78
120		0.77		0.77
110		0.76		0.77
100		0.74		0.76
90		0.71		0.75
Average ...		0.76		0.77
% Depression ...		24		23

Hence the depressing effect of K_2SO_4 is the same in presence or absence of acetaldehyde.

When, however, the ratios are calculated from 1 and 5 (extra phosphate in absence of acetaldehyde) and from 2 and 6 (extra phosphate in presence of acetaldehyde) it is found that they are very different in the two cases.

Depressing effect of phosphate.

Available phosphate in cc. of CO_2	Without acetaldehyde		With acetaldehyde	
	Ratio	$\frac{\text{Rate from 5}}{\text{Rate from 1}}$	Ratio	$\frac{\text{Rate from 6}}{\text{Rate from 2}}$
130		0.58		0.85
120		0.60		0.88
110		0.61		0.89
100		0.60		0.89
90		0.58		—
Average ...		0.59		0.88
% Depression ...		41		12

It is seen from these figures that the depression produced by extra phosphate is greatly diminished by the presence of acetaldehyde and it would hence appear that a large part of the depressing effect of phosphate is exercised on the production (or possibly the reduction) of the acceptor which is necessary for the attainment of a high phosphate rate [see Harden and Henley, 1920, 1921].

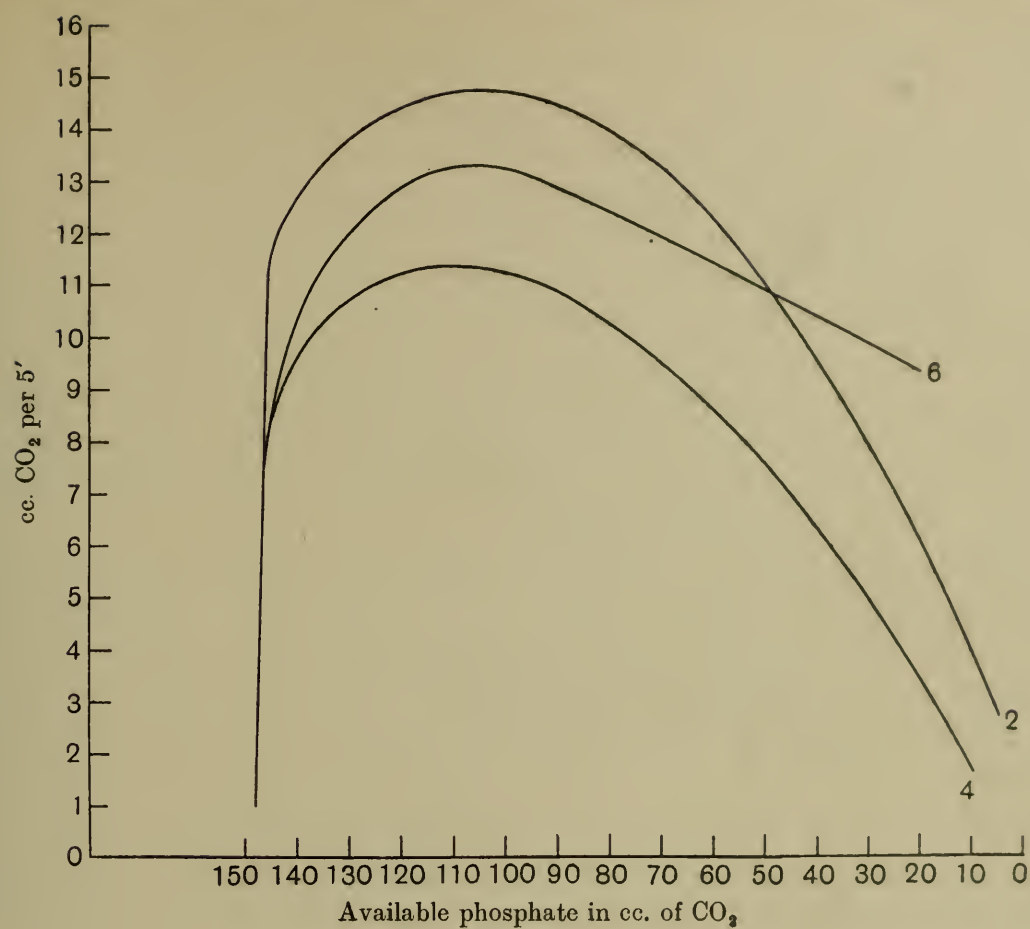


Fig. 1.

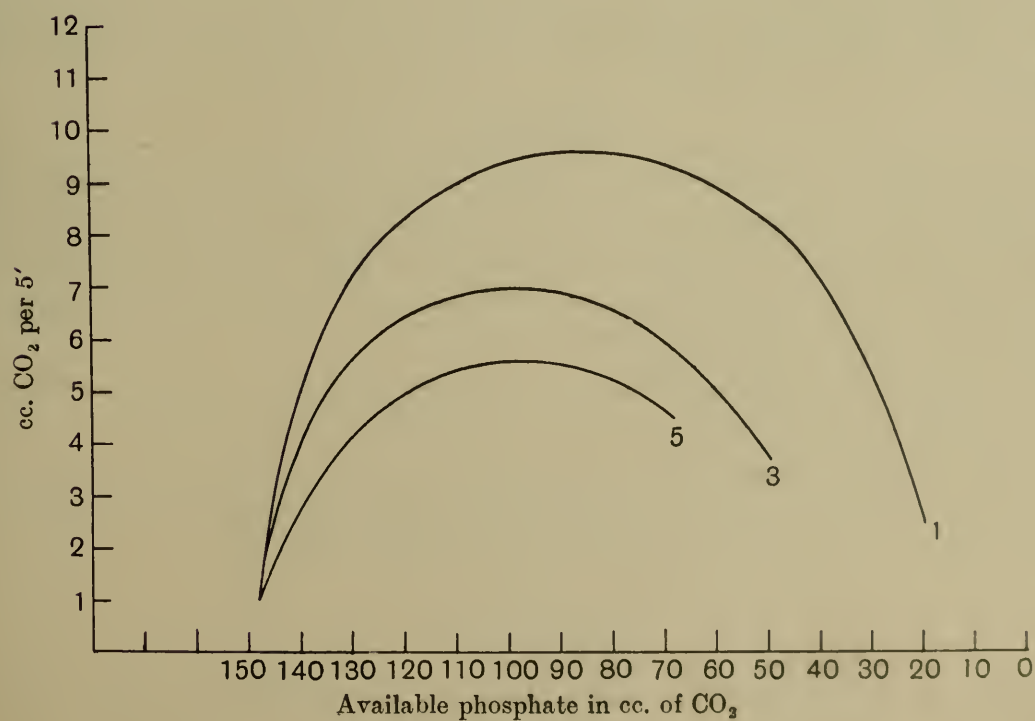


Fig. 2.

The sulphate on the other hand does not appear to have this specific action, since the effect is not altered by the addition of acceptor. The effect which it does exercise, and which may be regarded as the general salt effect of Meyerhof, appears to be a depression of the rate of one or more of the reactions in an early stage of the fermentation process, since, as we have seen, this salt is without any marked action on the decomposition of pyruvic acid by carboxylase. Whether the residual effect of phosphate, which is exercised in presence of acetaldehyde, is of the same nature or not cannot at present be stated [see Harden, 1914, p. 72]. It is interesting to note that whilst the total effect of phosphate in absence of acetaldehyde is much greater than that of sulphate, the residual effect in presence of acetaldehyde is much less. It is also worthy of note in this connexion that the normal function of phosphate is probably connected with one of these early stages.

Parallel experiments with glucose and yeast juice in which the concentrations of phosphate and sulphate were only half as great as in the experiments with fructose gave results in good agreement with those described above. The percentage depressions produced by K_2SO_4 were: in presence of acetaldehyde, 23 %; in absence of acetaldehyde, 26 %. The percentage depressions produced by K_2HPO_4 were: in presence of acetaldehyde, 17 %; in absence of acetaldehyde, 41 %. Since the ratio $\frac{\text{concentration of } K_2HPO_4}{\text{concentration of } K_2SO_4}$ is the same in both series of experiments and since the percentage depressions are approximately equal, it appears to follow that these salts affect the fermentation of glucose by yeast juice in a similar manner to that of fructose.

The salt effect seems to be correlated with the phosphate rate; and just as the maximum phosphate rate and tolerance of phosphate are greater with fructose than with glucose the tolerance of salt is also greater in the case of fructose.

SUMMARY.

1. As stated by Meyerhof, the chlorides and sulphates of sodium and potassium exert a depressing effect on the maximum rate obtainable and the rate of attainment of this maximum in the fermentation of glucose and fructose by yeast juice or zymine in the presence of phosphate. The effect of the sulphates is greater than that of the chlorides.

2. Salts diminish the rate of action of hexosephosphatase, but are without effect on that of carboxylase.

3. The depressing effects produced by potassium sulphate and by excess of phosphate differ in character, the latter being greatly diminished by addition of acetaldehyde, whereas the former is practically unaffected.

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FROM THE BIOCHEMICAL JOURNAL, VOL. XV, No. 3, 1921]

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L. THE SYNTHESIS OF VITAMIN B BY YEASTS (PRELIMINARY NOTE).

BY ARTHUR HARDEN AND SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute.

(Received May 5th, 1921.)

EXPERIMENTS on this subject have been in progress in this laboratory for a considerable time with the twofold object of ascertaining whether yeast grown on a medium devoid of vitamin B is able to produce this substance and whether different species of yeast all produce this vitamin. Our experiments are not complete but in view of the recent publication by Nelson, Fulmer and Cessna [1921] we think it desirable to make a brief statement of the results so far attained.

The method used consists in growing the yeast under examination on a synthetic medium containing ammonium phosphate and chloride as sources of nitrogen, together with the necessary mineral salts and cane-sugar. The cane-sugar was fractionally precipitated by alcohol from aqueous solution, and the solution of the dried purified material then shaken three times with fuller's earth to remove any possible trace of vitamin B.

The specimen of *S. cerivisiae* used had been isolated from a sample of baker's yeast, and *S. ellipsoideus* was also examined.

S. cerivisiae grew very slowly and imperfectly in the medium, whereas *S. ellipsoideus* grew much more rapidly and gave a larger yield.

The yeasts were centrifuged out of the medium, washed three times with distilled water, pressed and dried in the air. A parallel culture of *S. ellipsoideus* on unhopped brewer's-wort was made and the yeast crop treated in a similar manner.

The dried yeasts were then compared as regards their curative effect on pigeons suffering from avian polyneuritis as a result of a diet of polished rice, and showing retracted neck. Removal of this symptom for three days was regarded as a "standard" cure.

The following is a brief statement of the results.

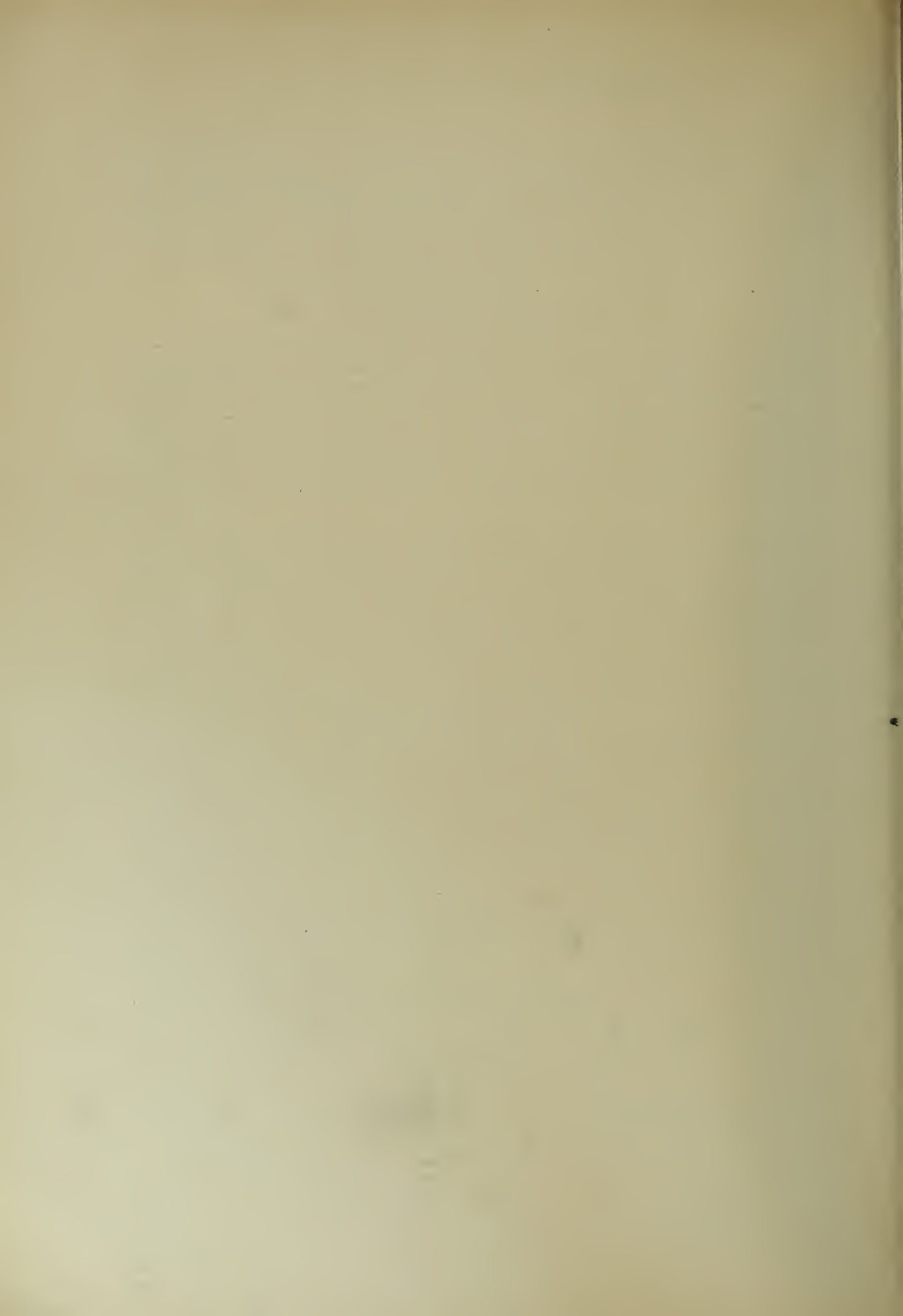
Material	Dose g.	Effect
Brewer's yeast	1	Cure
" " " " " "	0.5	"
<i>S. cerivisiae</i> from synthetic medium	1	Temporary cure, but recurrence after 48 hours
<i>S. ellipsoideus</i> from wort	1	Cure
" " " " " "	0.5	"
" " synthetic medium	1	" (lasting 4 days)
" " " " " "	1	" (" 6 ")
" " " " " "	0.5	Temporary cure, but recurrence after 48 hours

It appears therefore that *S. ellipsoideus* produces vitamin B and that the yeasts grown on the synthetic medium contain vitamin B, but not in so large a proportion as those grown on wort. Further experiments are in progress.

The expenses of this research were in part defrayed from a grant for which we are indebted to the Medical Research Council.

REFERENCE.

Nelson, Fulmer and Cessna (1921) *J. Biol. Chem.* **46**, 77.



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V. COMPARISON OF THE GROWTH-PROMOTING PROPERTIES FOR GUINEA-PIGS OF CERTAIN DIETS, CONSISTING OF NATURAL FOODSTUFFS.

BY ELEANOR MARGARET HUME.

From the Department of Experimental Pathology, Lister Institute.

(Received November 24th, 1920.)

INTRODUCTORY.

IN the course of researches upon guinea-pig scurvy, which have extended over several years, evidence has gradually accumulated which seems to show that the guinea-pig, for healthy growth and maintenance, requires an abundant supply of the fat-soluble *A* accessory factor of McCollum (vitamin *A* of Drummond [1920]). Two distinct lines of evidence support this conclusion.

The first line of evidence is derived from a number of experiments in which guinea-pigs grew on one group of diets and failed to grow on another group of diets. The distribution of vitamin *A* among foodstuffs has hitherto been only partially mapped out by the various workers in that field, yet, as far as the value for that factor of the various foodstuffs used is known, those which promoted growth seem to agree in containing vitamin *A* and those which did not, in lacking it. Furthermore those foodstuffs which produced partial growth seem to have done so in proportion to their known relative value for vitamin *A*. It is true that some of the diets which did not promote growth were open to criticism in some other respects than a deficiency of vitamin *A*, but it is also true that some diets on which growth took place were equally liable to have been deficient in the same particulars and yet promoted growth. The only dietary common factor, absent from the one set of diets and present in the other set, was vitamin *A*.

Most of the diets to be described were planned for the purpose of investigating the anti-scorbutic value of certain foodstuffs and in the very earliest experiments the need for vitamin *A* to be included in the basal diet was not recognised. It was, however, soon found that on many diets the animals did not thrive [see Chick and Hume, 1917], although they did not develop scurvy, and from this time onwards 60 cc. daily of milk (autoclaved at 120° for an hour, to destroy the anti-scorbutic factor present) was made a routine addition to most experimental scurvy diets; later on, as the result of added experience, the daily ration was often increased to 90 cc. for large and quickly growing guinea-pigs.

The second line of evidence is that derived from the histological examination of the rib junctions of all the experimental guinea-pigs, which was carried out as a routine by Miss F. M. Tozer. In the course of this examination she was forced to the conclusion that a bone lesion can occur on certain diets in which there can be no possible suspicion that the anti-scorbutic ration is insufficient. This bone lesion is in some stages indistinguishable from the bone lesion of scurvy and it occurs on many different diets which however have in common a deficiency in vitamin *A*. It is described by Miss Tozer [Delf and Tozer, 1918; Tozer, 1921]. The second line of evidence agrees with and supports the first in that the bone lesion is most severe where growth is least and in that failure in growth and severity of bone lesion both appear to be inversely proportional to the amount of vitamin *A* in the diet.

It is only proposed here to bring forward the facts which form the first line of evidence, *i.e.* to set out and compare the growth occurring in guinea-pigs on certain diets, differing from one another in their content of vitamin *A*. Miss Tozer's histological diagnosis of each case, wherever such was made, will at the same time be given for comparison, by her kind permission, but a full histological treatment of the whole question will be made by her separately in another communication.

Reference to the probable need of the guinea-pig for vitamin *A* has frequently been made in the papers of Dr Harriette Chick and her co-workers at the Lister Institute [Chick, Hume and Skelton, 1918; Delf and Skelton, 1918; Chick and Campbell, 1919]. Frölich [1912] refers to a fragility of the bones, attributed to a deficient diet, which is not scurvy. Jackson and Moore [1916] speak of a lesion resembling macroscopically a rachitic rosary, produced in guinea-pigs on a diet which was not quantitatively controlled but may have been deficient either in the anti-scorbutic or in vitamin *A* or in both. Hess and Unger [1918] also refer to changes in the rib junctions of guinea-pigs, on experimental diets, which they regard as more akin to rickets than to scurvy. It is thus apparent that a number of observers have noted the occurrence of more than one bone lesion in guinea-pigs, due to dietary causes, but there does not seem to be much evidence correlating any such lesion with a deficiency of vitamin *A*.

Cohen and Mendel [1918] conclude that the fat-soluble factor is not one of the primary factors concerned in scurvy of the guinea-pig, but do not state whether they consider it may have other importance.

TECHNIQUE.

The technique employed was the same as that which has been used throughout the Lister Institute scurvy experiments and has been detailed many times already. Young growing guinea-pigs about 320 g. weight were fed with a mixture of oats and wheaten bran *ad libitum*, but weighed. To this was added the food to be tested in weighed or measured quantities; the residues were either fed by hand or were measured and the quantity actually consumed

was calculated. In the present set of experiments, extra anti-scorbutic in the form of orange juice was added in those cases where the experimental ration did not already contain sufficient of that factor. It is proposed to give here details of various additions to this basal diet, together with details of the growth on such variously supplemented diets and a histological report, furnished by Miss Tozer.

The comparative growth of the animals has been set out in the form of a table, in which the weight at the start of the experiment and after 25, 35 and 45 days is given, together with the percentage increase for each animal and the average percentage increase for the whole group over each period. The normal as estimated by Delf [Delf and Tozer, 1918] is taken as the standard and in these experiments all degrees of deviation from it are found, down to cases such as Nos. 628, 629, 631, 632 (Table II, Group 28) on a diet of oats, bran, water and 10 cc. of orange juice daily, which showed no growth at all even in the first fortnight, but an actual loss in weight, amounting to an average of 19.7 % for the group for 25 days.

The histological bone lesion occurring in the rib junctions has been classified into the four very convenient degrees of severity already briefly described by Miss Tozer [Delf and Tozer, 1918]. She classifies the lesion occurring in guinea-pigs fed on a diet deficient in the anti-scorbutic or in vitamin *A* into four degrees of severity; normal, incipient, definite, acute; the exact significance of these terms can be ascertained on reference to her paper where diagrams are given. In some cases the experiments were continued for considerably longer than the 45 days for which the latest weight reading is given. In other cases the animal died after as short a period as 20 days so that the histological examination was made after the animals had been for very varying periods on the different diets. Doubtless this variation must affect to some degree the severity of the bone lesion but Miss Tozer has found that histological bone changes begin very early on a deficient diet [Tozer, 1921] and it is not therefore thought that such a variation seriously affects the comparative value of the histological findings, relative to one another. In the last column of the table the number of days elapsing between the beginning of the experiment and the date of death is noted.

Beyond the loss in weight and the bone lesion described, there are no other marked symptoms. The emaciation may be extreme, not a particle of fat being discoverable on the body in severe cases. All bones are more or less brittle and the animal is extremely prone to suffer from intercurrent disease, particularly lung affections.

EXPERIMENTAL RESULTS.

The experimental results can be divided roughly into two groups, those concerned with foods which promoted growth (Table I) and those concerned with foods which did not (Table II). Naturally the division is only an artificial one, for many of the foodstuffs not promoting growth might promote

growth if given in much larger quantities and many of the growth-promoting ones would fail to do so if given in much smaller quantities, but for purposes of convenience such a division is permissible. The first or growth-promoting group includes milk in several forms, green cabbage or some modification thereof and hay. The second or non-growth-promoting group includes white cabbage and a derivative thereof, swede juice, boiled onion, orange juice and germinated peas.

Green cabbage leaf, raw. (Table I, Groups 1, 2 and 3.)

Group 1. 30 g. daily ration. An oat and bran diet to which 30 g. daily of raw green cabbage is added is richly growth-promoting and the average percentage increase for a group of animals for 45 days was 60.4 %, actually more than the normal standard of 55.8 %, calculated for the same period.

Histological examination of the costo-chondral junctions of the ribs. Only two animals were examined, after 39 days of the experiment, and both showed a normal histology.

Group 2. 15 g. daily ration. With a 15 g. daily ration, good growth but not to the full normal extent is induced. The average percentage increase for a group of seven animals for 45 days being 40.1 % which is only about two-thirds of that on 30 g.

Histological examination. Only four animals were examined; three were quite normal and the fourth was nearly so, not being sufficiently abnormal to be classed as "incipient."

Group 3. 5 g. daily ration. A ration of 5 g. daily also produced growth, but the increase was still further below the normal, being an average of only 29.9 % for a group of three animals for 45 days, *i.e.* about half the corresponding figure for 30 g.

Histological examination. The three animals in this group were all examined; one was not quite abnormal enough to be called incipient and the other two were between incipient and definite.

Green cabbage leaf, heated for one hour at 100°. (Table I, Groups 4 and 5.)

The cabbage was prepared by being steamed for an hour at 100°.

Group 4. 30 g. daily ration. On a 30 g. daily ration a group of three animals showed a rate of growth equal to that on 30 g. raw cabbage. The two animals on which the experiment was continued for 45 days showed an even better average rate of increase (71.4 %) than the rate of increase for 30 g. raw cabbage (60.4 %).

Histological examination. All three animals were examined, two of them were found to be perfectly normal and the third showed a very slight departure from normality but not sufficient to be called abnormal.

Group 5. 15 g. daily ration. Three animals received a daily ration of 15 g. and showed a rate of growth (45.5 %) slightly superior to that (40.1 %) for 15 g. of raw cabbage.

Table I.

Group no.	Additions to basal diet of oats, bran and water	Amount of ration	No. of animal	Initial weight g.	Wt after 25 days g.		% increase	Wt after 35 days g.		% increase	Wt after 45 days g.		% increase	Histology			Length of life from beginning of expt. to post-mortem, days
														Normal	Incipient	Definite	Acute
1	Normal	340	450	...	32.3	500	...	47.0	535	...	55.8				...
	Green cabbage leaf, raw	30 g.	1	285	380	...	33.3	415	...	45.6	470	...	64.9	Not examined			...
	"	"	8	280	365	...	29.8	415	...	48.2	475	...	69.6	"			...
	"	"	351	368	470	...	27.7	490	...	33.1	520	...	41.3	"			...
	"	"	352	338	480	...	42.0	540	...	59.7	565	...	60.5	"			...
	"	"	885	340	440	...	29.3	480	...	41.1	510	...	50.0	"			...
	"	"	150	325	477	...	46.7	541	...	66.4	572	...	76.0	"			...
	"	"	152	315	440	...	39.6	473	...	50.1	"			39
	"	"	153	330	418	...	26.6	458	...	38.7	"			39
	Average	Average	34.2	47.8	60.4				...
2	Green cabbage leaf, raw	15 g.	359	350	470	...	34.2	480	...	37.1	480	...	37.1	Not examined			...
	"	"	353	345	443	...	28.4	472	...	33.9	495	...	43.4	"			...
	"	"	886	310	340	...	9.7	370	...	19.3	400	...	29.0	"			...
	"	"	826	340	440	...	29.4	460	...	35.2	515	...	51.4	"			...
	"	"	823	370	445	...	20.2	450	...	21.6	495	...	33.7	"			93
	"	"	70	330	435	...	30.4	485	...	46.9	510	...	55.5	Not examined			...
	"	"	71	305	390	...	27.9	375	...	22.9	400	...	31.1	Not examined			71
	"	"	699	321	388	...	20.8	425	...	32.4	"			38
	"	"	693 A	330	348	...	5.4	"			25
	Average	Average	22.9	31.1	40.1				...
3	Green cabbage leaf, raw	5 g.	421	330	378	...	14.5	390	...	18.1	420	...	27.2	"			94
	"	"	422	320	340	...	6.2	362	...	13.1	403	...	25.9	"			94
	"	"	425	318	355	...	11.6	398	...	25.1	435	...	36.7	"			94
	Average	Average	10.8	18.7	29.9				...
4	Green cabbage steamed 100°C. 1 hr.	30 g.	333	330	465	...	40.9	525	...	59.0	580	...	75.7	"			84
	"	"	334	305	395	...	29.5	455	...	49.1	510	...	67.2	"			84
	"	"	335	315	392	...	23.1	435	...	38.0	"			86
	Average	Average	31.2	48.7	71.4				...
5	Green cabbage steamed 100°C. 1 hr.	15 g.	338	300	385	...	28.3	440	...	46.6	465	...	55.0	"			102
	"	"	339	330	395	...	19.7	438	...	32.7	460	...	39.3	"			...
	"	"	340	350	405	...	15.7	465	...	32.8	498	...	42.2	"			...
	Average	Average	21.2	37.3	45.5				...

6	Green cabbage steamed 100°C. 15 g. 2 hrs.	354 355 356 357 358	320 330 335 340 330	400 398 413 415 435	25.0 20.6 16.5 22.0 31.8	430 430 442 430 452	34.3 30.3 24.5 26.4 39.9	452 448 470 470 480	38.1 35.7 32.3 38.2 45.4	+	96 90 95
			Average	...	23.2		30.4		37.9					
7	Raw cabbage juice, filtered ... 20 cc.	CJ 1 CJ 2	338 332	380 350	18.3 5.4	425 380	25.7 14.4	454 380	24.3 14.4	+nearly +	108 109
8	Raw cabbage juice, filtered ... 30 cc.	CJ 10	331	410	23.8	430	29.9	440	32.9	...	+	to	+	100
9	Raw cabbage juice, unfiltered 30 cc.	CJ 9	335	403	19.4	425	26.8	440	31.0	...	+	to	+	100
10	Heated cabbage juice, filtered 20 cc.	CJ 5 CJ 6	329 332	370 351	12.4 5.7	316 369	- 3.9 + 11.1	292 345	- 11.2 + 3.9		Not examined
	"		Average	...	9.0		3.6		- 3.6	
11	Heated cabbage juice, filtered 20 cc.	CJ 4	330	355	7.6	303	- 8.1	342	3.6		Not examined
	+ 2 g. casein		Average	...	7.6		- 8.1		3.6					
12	Heated cabbage juice, filtered 30 cc.	CJ 3	330	400	21.2	428	29.6	456	38.3		Not examined
			Average	...	21.2		29.6		38.3					
13	Heated cabbage juice, un- filtered	CJ 7 CJ 15	335 345	380 398	13.4 15.3	395 435	16.7 26.0	312 420	- 6.8 21.7	...	Not examined	+	to	...
	"		Average	...	14.3		21.3		14.9					56
14	Heated cabbage juice, un- filtered + 2 g. casein	CJ 8	331	415	25.3	435	31.4	431	30.2	+	...	64
			Average	...	25.3		31.4		30.2					
15	Heated cabbage juice, un- filtered	CJ 11 CJ 12 CJ 13 CJ 14 CJ 18	332 333 332 339 340	400 409 387 413 314	20.5 22.8 13.5 21.8 ...	404 422 389 440 355	21.6 26.7 17.4 29.7 ...	393 450 400 460 375	18.3 35.1 20.4 35.6 ill and re- covered	...	+	59 121 120 115 62
	"		335	403	20.1	435	29.8	422	25.9	...	+	to	+	94
	"	CJ 21	345	370	7.2	395	14.4
		Average	17.6		23.2		27.0					
	50 cc.	CJ 21	398	15.3	+	nearly	49
							Average	...	15.3					

Table I continued

Group no.	Additions to basal diet of oats, bran and water	Amount of ration	No. of animal	Initial weight g.	Wt after 25 days g.		% increase	Wt after 35 days g.		% increase	Wt after 45 days g.		% increase	Histology				Length of life from beginning of expt. to post-mortem, days
														Normal	Incipient	Definite	Acute	
16	Normal	340	450	...	32.3	500	...	47.0	535	...	55.8	...	+	85
	Dried cabbage juice, green	50 cc.	DC 1	301	360	...	19.9	390	...	29.5	395	...	31.2	...	+	91
	+ 5 cc. orange juice	"	DC 2	313	370	...	18.2	333	...	6.3	347	...	11.1	...	+	82
	"	"	DC 3	304	350	...	15.1	360	...	18.4	365	...	20.0	+	to	
	Average	Average	17.7	18.0	20.7	
17	Fresh milk + 10 cc. orange juice	20 cc.	684	334	305	...	- 8.6
	"	"	685	335	350	...	+ 4.4
	Average	Average	- 2.1
18	Raw milk + 10 cc. orange juice	60 cc.	699 M	330	450	...	36.3	485	...	46.9	480	...	45.4	+	45
	"	"	699 N	333	410	...	23.1	429	...	28.8	456	...	36.9	+ nearly	+	45
	"	"	699 O	338	440	...	30.1	489	...	44.7	532	...	57.4	+ nearly	+	45
	"	"	699 P	331	424	...	28.9	470	...	41.9	490	...	48.0	+ nearly	45
	Average	Average	29.6	40.5	46.9
19	Aut. milk + 10 cc. orange juice	60 cc.	699 Q	331	455	...	37.4	516	...	55.9	532	...	60.7	+ nearly	45
	"	"	699 R	330	440	...	33.3	470	...	42.4	500	...	51.5	+ nearly	45
	"	"	699 S	340	427	...	25.5	445	...	30.8	445	...	30.8	...	+	45
	"	"	699 T	340	428	...	25.8	463	...	36.1	464	...	36.4	+ nearly	45
	Average	Average	30.5	41.3	44.8
20	Glaxo = 60 cc. raw milk + 10 cc. orange juice	60 cc.	694	337	441	...	30.8	466	...	38.2	474	...	40.6	...	+	to	...	45
	"	"	695	334	423	...	26.6	453	...	35.9	480	...	43.7	...	+	45
	"	"	696	330	407	...	23.1	435	...	31.8	435	...	31.8	...	+	to	...	45
	"	"	697	334	403	...	20.6	417	...	24.9	450	...	34.7	...	+	45
	Average	Average	25.2	32.7	37.7

Histological examination. Only one animal was examined and was found nearly normal.

Steaming cabbage for an hour at 100° does not therefore appear to destroy a perceptible amount of its growth-promoting powers for guinea-pigs.

Green cabbage leaf, heated for two hours at 100°. (Table I, Group 6.)

The cabbage was prepared by being steamed for two hours at 100°.

Group 6. 15 g. daily ration. Five animals received 15 g. daily each and their average percentage increase (37.9 %) for 45 days, is only slightly inferior to the corresponding figure for raw cabbage (40.1 %).

Histological examination. Three of the five animals were examined and showed a normal histology.

Cabbage steamed for two hours at 100° does not appear to be materially damaged as regards its growth-promoting powers for guinea-pigs.

Filtered green raw cabbage juice. (Table I, Groups 7 and 8.)

A number of experiments were planned in order to dissociate the growth-promoting fraction, as far as possible from the proteins and other substances in the actual tissues of the leaf. Juice from raw and heated cabbage, filtered and unfiltered, was used and in one or two cases caseinogen was added to compare the effect.

For the preparation of the raw juice the green portions of the leaf, free of the mid-rib, were minced in a mincing machine, the pulp was enclosed in muslin and pressed out in a hand press. In all cases the animals were allowed to drink the juice if they would; if they would not it was fed by hand. The juice was filtered through paper.

Group 7. 20 cc. daily ration. Two animals received 20 cc. of the raw juice filtered and made some growth, *i.e.* an average of 24.3 % in 45 days; this is a rate of increase well below the normal (55.8 %) and less even than that (29.9 %) made on 5 g. daily of the raw green leaf.

Histological examination. Both animals were examined and one was found to be almost completely normal and the other between normal and "incipient."

Group 8. 30 cc. daily ration. One animal received 30 cc. daily and showed 32.9 % increase in 45 days, an increment which is still nearly 50 % below the normal.

Histological examination. The condition of the rib junctions was distinctly abnormal, being between "incipient" and "definite."

Raw filtered cabbage juice is thus capable of promoting growth but possesses this property in a much less degree than the raw leaf.

Unfiltered green raw cabbage juice. (Table I, Group 9.)

The juice used was the same as in Groups 7 and 8 except that it was unfiltered.

Group 9. 30 cc. daily ration. Only one animal received this ration and its percentage increase for 45 days, *i.e.* 31 %, was almost identical with that

(32.9 %) of the one animal (Group 12) which received 30 cc. of the filtered juice.

Histological examination. The histological result also corresponded with that of the animal in Group 12, being between "incipient" and "definite."

So far as evidence derived from comparison between two single animals is worth anything at all, it points to there being no loss of growth-promoting power in cabbage juice on filtration through paper.

Filtered green heated cabbage juice. (Table I, Groups 10, 11 and 12.)

The juice was prepared by steaming the cabbage at 100° for 20 minutes, folding it in muslin and pressing in a hand press. It was then filtered through a paper filter.

Group 10. 20 cc. daily ration. 20 cc. of the juice was fed to two animals which both showed some rise in weight at the 25th day (average 9.0 %) but this had diminished to 3.6 % on the 35th day. On the 45th day one of the two animals showed a percentage loss over the whole period of 11.2 % while the other showed some loss of what it had previously gained, though there was a net gain over the whole period of 3.9 %. The gain for this animal (No. CJ 6) for 35 days was 11.1 %.

No histological examination was made.

Group 11. 20 cc. juice + 2 g. caseinogen daily ration. The caseinogen was unpurified; it was mixed with a little water and was fed by hand. The quantity 2 g. was selected as being somewhat in excess of the amount present in 60 cc. of cow's milk, a ration of milk well known to be growth-promoting for guinea-pigs.

One animal received the ration but the caseinogen did not affect the result in its case for it just showed maintenance over the whole period of 45 days.

No histological examination was made.

Group 12. 30 cc. daily ration. Only one animal received 30 cc. of the filtered heated juice; for the period of 45 days it showed considerable growth (38.3 %), not equal to the normal (55.8 %), but nearly approaching that for 15 g. raw green cabbage leaf (40.1 %), and rather greater than that for 30 cc. of the raw juice either filtered or unfiltered.

No histological examination was made.

On the 20 cc. dose the juice of heated cabbage appears to be much inferior in growth promoting power to the raw juice, but on the 30 cc. dose this difference is not apparent; individual idiosyncrasy is however so great that very little importance can be attached to the behaviour of single animals, such as are available for comparison of the 30 cc. doses.

Unfiltered green heated cabbage juice. (Table I, Groups 13, 14 and 15.)

The juice was prepared as for Groups 10, 11 and 12 but it was not filtered.

Group 13. 30 cc. daily ration. Two animals received a 30 cc. daily ration

and both of them made about half the normal growth for 35 days, but by the 45th day one animal, CJ 15, had begun to lose slightly and the other, CJ 7, had converted the previous gain into a negative balance of 6.8 %. In such cases as this the dose would appear to be sufficient to start growth but ceases to be so when the animal is larger. It can scarcely be that the animal starts growth on a reserve of its own which gradually becomes exhausted, as seems to be the case with the rat, when suffering from a deficiency of vitamin A, because on some of the diets, such as oats and bran and orange juice (Table II, Group 28), there is absolutely no increase in weight at all but a rapid decline from the start.

Histological examination. The one animal, CJ 15, which made the better growth gave a histological result between "incipient" and "definite"; the other animal was not examined.

Group 14. 30 cc. juice + 2 g. caseinogen, daily ration. This group only contained one animal which made rather better growth than the two animals in the preceding group; at 35 days it showed an increase of 31.4 % but by the 45th day it also had begun to decline and showed a net increase for the whole period of only 30.2 %.

Histological examination. The lesion in the rib junctions was of the third degree, being "definite."

It is unlikely that the rather better growth shown in this case on the addition of caseinogen was due to more than the individual idiosyncrasy of the animal; the addition of caseinogen in Group 2 produced no such effect.

Group 15. 40 cc. daily ration. This group includes seven animals, of which one was ill during the experiment but recovered, the values for it are therefore given but are not included in the averages. The group for each of the three time periods shows a rate of increase about half the normal.

One animal (CJ 21) which showed a low rate of growth had the daily dose increased to 50 cc. after the 35th day but this increase was not reflected in the subsequent rate of growth although the histology was almost perfectly normal.

Histological examination. The histological results were better than those recorded for any of the other cabbage juice groups, excepting Group 7 (20 cc. raw green filtered juice). One animal showed an almost normal histology, three were between normal and "incipient," one was "incipient," one was between "incipient" and "definite" and one was "definite."

Taken together the cabbage juice results indicate that filtration through paper does not affect the growth-promoting properties of cabbage juice but that the juice from the raw leaves is more potent than that from the cooked leaves. As it was observed, in experiments already detailed, that steaming did not appreciably diminish the value of the leaves when fed whole, it must be that the growth-promoting principle does not pass out with the juice on pressure as readily after heating as it did before. Perfectly normal growth was not obtained in any case with the cabbage juice rations but the indica-

tions are that such growth would be secured if larger doses of the juice could be conveniently given to the animals.

Dried cabbage juice. (Table I, Group 16.)

The juice was prepared from steamed cabbage leaves in the manner already described but was not filtered. The yield of juice was about 50 % of the weight of cabbage taken. It was poured into large flat dishes which were placed in a hot room (37°) with an electric fan just beside them. After about 24 hours the liquid had evaporated to a sticky, thick paste and was further dried by the method described by Harden and Robison [1919]. The paste was scraped up and worked up with a knife so as to become full of air bubbles. The aerated mass was placed in a desiccator which was then evacuated. The whole mass swelled and rose up and if left in the desiccator was dry and porous by the next morning and could be broken up into a coarse powder. Frequently however the drying was not complete in one operation and it was necessary to work the mass up and evacuate the desiccator several times. The dry solids generally amounted to about 5 % of the weight of juice taken. The powder was extremely hygroscopic and had to be kept in a desiccator; new batches were made about once a fortnight. For administration to the guinea-pigs, the powder was mixed with a little water and given by hand.

With this ration 5 cc. of orange juice was given daily, as the anti-scorbutic value of the cabbage juice was likely to have suffered seriously in the process of drying.

Group 16. 50 cc. daily ration. The dried cabbage juice was fed to three guinea-pigs in amounts calculated to be equivalent to 50 cc. of the heated juice. Growth was obtained but was less than one-half the normal and was also less than that obtained on smaller doses of the heated juice which had not been dried.

Histological examination. The results were also rather inferior to those on smaller doses of the heated juice. One animal was between normal and "incipient" and two were "incipient."

There is some loss of growth-promoting power on drying cabbage juice but the loss is by no means complete.

Raw cow's milk. (Table I, Groups 17 and 18.)

The milk used in these experiments was very pure, freshly delivered certified country milk; the time of year at which the experiments were performed was unfortunately not the same in every case but the months are given in each case. 10 cc. orange juice daily was given with this and all other milk rations, to supply the anti-scorbutic factor which would otherwise have been insufficient.

Group 17. 20 cc. daily ration, increasing to 40 cc. and later to 60 cc. Dec.-Feb. Two animals received 20 cc. daily and after 25 days one, No. 684, showed a loss of 8.6 % and the other, No. 685, showed a gain of 4.4 %.

After 30 days the amount of milk was increased to 40 cc. daily and after 30 days on this altered ration No. 684 had recovered a very little weight but had not regained its original weight. No. 685 had gained steadily, though not normally, and was allowed to remain on the 40 cc. ration on which it continued to gain slowly for over 80 days. No. 684, however, was altered to 60 cc. daily on which after a little delay it made a sharp gain in weight.

No histological examination was made.

From experiments on the anti-scorbutic values of foodstuffs published elsewhere [Chick and Hume, 1917] it is well known that 60 cc. of autoclaved milk daily gives very good, if not perfectly normal growth in guinea-pigs, when added to a diet of oats and wheaten bran and orange juice. The foregoing experiment was sufficient to orientate and to show that a quantity less than 60 cc. of raw milk will not do so. This amount was therefore chosen as the daily ration in the following experiments, Groups 18, 19, 20 and 21, in which the growth-promoting powers of several kinds of milk were examined.

Group 18. 60 cc. daily ration. June-July. Four animals received 60 cc. daily of the raw milk and made good but not quite normal growth, the average increase for the group for 45 days being 46.9 % as against 55.8 % for the normal.

Histological examination. Of the four animals one was perfectly normal and the remaining three were almost completely so.

It would therefore appear that 60 cc. raw milk a day is almost but not quite a sufficient quantity to promote normal growth.

Cow's milk autoclaved for one hour at 120°. (Table I, Group 19.)

The same milk as that used in Groups 17 and 18 was autoclaved for one hour at 120°.

Group 19. 60 cc. daily ration. June-July. Four animals receiving the above ration showed a rate of growth (44.8 %), almost identical with that (46.9 %) of the animals which received 60 cc. raw milk.

Histological examination. The results were very slightly inferior to those of animals in Group 18, three being nearly normal and one "incipient."

It therefore appears that heating for an hour at 120° in an autoclave does not appreciably damage the growth-promoting power of milk for guinea-pigs.

Dried milk. (Table I, Group 20.)

A well-known brand of dried milk, much used in infant feeding, was employed. It was not more than a month old and was prepared in March and April. The milk was made up so that the liquid should contain 3.5 % of fat but the powder is a proprietary article in which the original composition has been slightly modified. No fine quantitative deductions could therefore be drawn from the result, but it should be possible to say whether drying seriously damages the growth-promoting properties of milk for guinea-pigs or no.

Group 20. 60 cc. daily ration. Four animals received the 60 cc. daily ration and showed over a period of 45 days a rate of growth (37.7 %), slightly inferior to the corresponding rate (46.9 %) for raw milk.

Histological examination. The results were distinctly inferior to those on raw milk, two animals being "incipient" and two between "incipient" and "definite."

Since the dried milk was not prepared from the milk used in the raw milk experiments and since the time of year and consequently the food of the cows were different, it is not possible to attribute the slight inferiority of the dried milk, with certainty, to the fact that it had been dried. It is possible however to say that if drying affects the growth-promoting power of milk for guinea-pigs at all, it only does so very slightly.

Hay autoclaved at 120° for one hour.

No continuous experiments were put up with a daily ration of hay, but six animals receiving oats and bran and varying rations of lemon juice, and ten animals receiving oats and bran and varying rations of germinated peas showed no appreciable growth. Autoclaved hay (about 20 g. daily) was then added to the diets for four days and every animal showed an immediate sharp rise in weight which at once ceased when the hay was taken away again.

After drying and autoclaving for an hour at 120°, therefore, hay still retains considerable growth-promoting power for guinea-pigs, though the above observation gives no indication whatever, whether some of the original growth-promoting power is lost or not.

White cabbage leaf, raw. (Table II, Group 21.)

The first of the non-growth-promoting group of foodstuffs is the raw white heart of cabbage. Very tightly closed cabbage hearts were used and the outer green leaves were stripped off until inside leaves of a uniform pale etiolated greenish yellow were reached.

Group 21. 15 g. daily ration. Guinea-pigs do not willingly eat the heart leaves of cabbage but two were persuaded to do so. One, No. 698, failed altogether to grow and when the experiment ended after 39 days showed a loss of 4.3 %. The other, No. 692, showed a gain of 4.4 % at the 35th day, which was converted into a loss of 2.4 % by the 45th day.

Histological examination. The lesion was well marked being between "incipient" and "definite" for one animal and "definite" for the other.

Since even as little as 5 g. of green raw cabbage have been shown to produce as much as half the normal growth, while 15 g. of white cabbage produce practically no growth at all, it is clear that white cabbage is vastly inferior to green in its growth-promoting properties. When however the state of affairs on a white cabbage ration is compared with that on oats, wheaten bran and orange juice it is apparent that the white cabbage cannot be so completely deficient in growth-promoting properties as is the orange juice. The

former at least secures something like maintenance for as much as 45 days, whereas on the orange juice there is a heavy loss (average 19.7 %, Group 28, Table II), even in 25 days.

Unfiltered heated white cabbage juice. (Table II, Group 22.)

The juice was prepared in the same way as the juice of steamed green cabbage leaves and the leaves were selected as in the foregoing experiment (Group 21). The juice was not filtered.

Group 22. 40 cc. daily ration. Three animals received the above ration; of these one showed no growth but a negative balance of 4 % even by the 25th day; on the 35th day the negative balance was increased to 12.5 % and before the 45th day the animal was dead. The other two animals, CJ 16 and CJ 17, showed some increase (9 and 10 %) on the 25th day, but by the 35th day CJ 17 was losing weight and was dead before the 45th day; CJ 16 went on gaining a little to the 25th day (14.6 %) but by the 45th day showed a negative balance of 4 % and died shortly afterwards.

Histological examination. One animal was between "incipient" and "definite," one "definite" and one between "definite" and "acute."

Comparison of the 40 cc. dose of the green (Group 15) and of the white cabbage juice shows the great inferiority of the latter. On the green, growth amounting to about half the normal was obtained; on the white, after slight initial growth the animals declined and died in 45 days or less.

Steamed Onion. (Table II, Groups 23 and 24.)

The onion was steamed in a double saucepan for a rather indeterminate time, probably about one hour; it is however known from the experiments with steamed cabbage that steaming even for two hours is unlikely greatly to damage the growth-promoting property if present.

Group 23. 30 g. daily ration. Four animals received this ration and did not give a very uniform result. Two, Nos. 62 and 63, showed a considerable rise, nearly half the normal in the first 25 days but one of these declined and was dead very soon after. No. 63 increased a little further to the 35th day (23.3 %) but then began to decline very slowly, showing a net increase of only 20 % on the 45th day. The other two, Nos. 64 and 65 A, both showed a constantly increasing loss amounting to 10 % in one case and 37.8 % in the other, by the 45th day.

Histological examination. Three of the five animals were examined; one was between "incipient" and "definite" and two were "definite."

Group 24. 60 g. daily ration. One animal received a daily ration of 60 g. on which it showed no gain but almost maintained itself for 45 days.

Histological examination. The lesion was "definite."

Steamed onion does not seem to be wholly devoid of growth-promoting power for guinea-pigs but in most cases even in large rations it produces very little effect.

Swede juice. (Table II, Group 25.)

The juice of the swede turnip (*Brassica campestris*, var. *Napo-Brassica*) was expressed by first grating the swede root on a kitchen grater and then enfolding the pulp in muslin and squeezing out the juice by hand.

Group 25. 20 cc. daily ration. Four animals received the ration and three showed a decline from the start; of these two were dead by the 45th day. The fourth, No. 539, showed an increase of 10.1 % on the 25th day, but by the 35th day the net increase was only 7.6 % and by the 45th day there was a negative balance of 4.3 %.

Histological examination. One animal was "incipient" and three "definite."

Swede juice is evidently almost devoid of growth-promoting power for guinea-pigs in quantities of 20 cc. daily.

Germinated peas. (Table II, Groups 26 and 27.)

Peas were germinated by soaking in water for 24 hours and germinating them in a funnel for 48 hours; the outer coat of the seed was removed and the peas fed to the guinea-pigs whole.

Group 26. 20 g. daily ration. Three animals received the ration for 25 days and just maintained weight; only one received it for the full 45 days and showed a very slight negative balance.

No histological examination was made.

Group 27. 30 g. daily ration. Two animals were used but the experiment only lasted 20 days in one case and 23 in the other, both animals showed slight negative balance.

No histological examination was made.

Germinated peas appear to possess very little growth-promoting power for guinea-pigs.

Orange juice. (Table II, Group 28.)

The oranges were cut across and the juice expressed by hand.

Group 28. 10 cc. ration daily. Four animals received the ration and after 25 days showed a large negative balance, amounting to an average for the group of 19.7 %.

Histological examination. The lesion was very bad, three animals being "definite" and one "acute."

The dose of orange juice given was only a small one, not sufficient to amount to a real test of the growth-promoting properties of orange juice; the result with these four animals is only quoted here to show that there is no gain at all but an immediate and rapid loss on the most deficient type of diet.

DISCUSSION AND CONCLUSIONS.

It is believed that the foregoing experiments indicate that the guinea-pig requires for growth and maintenance a considerable quantity of the fat-soluble accessory factor of McCollum (vitamin *A* of Drummond) in its diet.

Such a conclusion is reached for two reasons, firstly the diets which produced growth were not superior in their protein, salt or caloric value, to those which did not; the difference must therefore lie in their vitamin content, but it is known that the anti-scorbutic and anti-neuritic vitamins were adequately supplied in all cases; the only known dietary constituent which could have been lacking is thus the vitamin *A*.

Secondly, the distribution of the growth-promoting factor revealed in these experiments, and its behaviour under certain forms of treatment, correspond with what is known of the distribution of vitamin *A* and its behaviour in similar circumstances.

To take the first point in more detail, growth occurs on green cabbage juice but not on white nor on swede juice, it occurs on green cabbage leaf (5 g.) but not white (15 g.) nor on germinated peas nor on onions. These sets of rations do not differ materially from one another in salt, protein or caloric value, the difference must rather be that of a body, present only in the minute amounts in which a vitamin is present. If it be granted then that the growth-promoting factor in this set of experiments is probably a vitamin, it is further apparent from the next paragraph that that vitamin corresponds with vitamin *A* in its distribution among foodstuffs and in its behaviour.

The work of McCollum has shown that vitamin *A* is present in green leaves [McCollum, Simmonds and Pitz, 1916] and in the butter fat of milk [McCollum, Simmonds and Pitz, 1916; McCollum and Davis, 1919]; in his book, McCollum [1920, p. 89] makes the generalisation, drawn from various experiments, that it is only present to a small extent in most roots and non-fatty seeds, such as cereals. Osborne and Mendel [1919] show that it is still present in green leaves after drying. Correspondingly it is found in the experiments detailed in this paper, that green cabbage and milk and dried leaves (hay) promote growth in guinea-pigs, while roots, as represented by swede juice and onions (truly an underground stem), and seeds, as represented by peas, fail to do so.

Coutts and Winfield [1918] have shown that milk, after drying, is still a rich source of vitamin *A* for rats and similarly in these experiments, dried milk is found to be little if at all inferior to raw milk in its growth-promoting properties.

Till recently the evidence with regard to the behaviour of vitamin *A* towards heat was conflicting but the position is now greatly cleared up by a contribution of Hopkins [1920], which shows that vitamin *A* is resistant to heat only as long as there is no access of oxygen but that as soon as oxidation is permitted at the time of heating, destruction of vitamin *A* begins to take place. In the present experiments in which milk and cabbage were heated in an autoclave or in a steamer, little or no loss of growth-promoting properties could be detected and correspondingly it is clear that the conditions were not such as would promote oxidation.

Lastly Drummond and Coward have found (Unpublished Experiments) that etiolated and non-green organs of plants are deficient in vitamin *A*,

corresponding with the present results that they are non-growth-promoting for guinea-pigs.

The correspondence is complete as far as the experiments go and there are no discordant facts, but on the other hand the evidence is not quite complete, for in no case can it be said that the *only* difference between a growth-promoting and a non-growth-promoting diet was the presence or absence of vitamin *A*; were it shown for instance that milk on heating with access of oxygen loses at the same time its growth-promoting power for guinea-pigs and its vitamin *A* value for rats, the evidence could be regarded as conclusive.

Prima facie it is extremely probable that the guinea-pig would require vitamin *A* for it is akin to the rat and must be accustomed, in natural conditions, to a diet of greenstuff even richer in vitamin *A* than the diet of the wild rat; such a line of argument is not however really permissible, for the guinea-pig differs, on the other hand, very markedly from the rat in its large need of the anti-scorbutic factor, a need so large that it has probably limited the creature's geographical distribution and made the numerical success achieved by the rat, mouse and rabbit in the struggle for existence, an impossibility for it.

If it can be accepted from these experiments that the guinea-pig is an animal requiring a large supply of vitamin *A*, it provides another animal on which the vitamin *A* value of certain classes of foodstuffs can be tested. The technique of rat feeding for testing the value of substances in vitamin *A* has now been much improved by the work of Drummond and Zilva but it still holds many difficulties and when the vitamin *A* value of a substance is low, so that large quantities of it have to be given, the technique sometimes fails altogether and frequently from the quantitative point of view. It is exceedingly difficult to hand-feed an unwilling rat, it is comparatively easy to do so with a guinea-pig. Further, guinea-pigs do not appear to grow at all if the diet is entirely deficient in vitamin *A*, though perhaps a larger number of experiments is needed, before this can be affirmed absolutely to be the case. If, as is believed, it is the case, then the growth achieved on any diet can be regarded as a measure of the amount of vitamin *A* in that diet and even the first few days of growth or failure can be regarded as the true trend of the whole of the rest of the experiment, without the need of any preliminary depletion period such as it is often customary to use with rats. In this way, vegetables, fruits, extracts of substances and other non-fatty preparations can be tested on guinea-pigs for their vitamin *A* value, but, as far as can be seen, fats cannot be so tested. Fat as emulsion in milk is tolerated by the guinea-pig but for more concentrated fats it appears to have an intolerance; cream and egg yolk were found to be fatal and codliver oil, even in small doses, appeared to have a very deleterious effect; these substances are however ones with which the rat technique is able to deal.

The above experiments were all conducted under the direction of Dr

Harriette Chick by whom I am kindly permitted to collect and tabulate them. Almost every one of those who have ever cooperated with Dr Chick, in her work on deficiency diseases, has contributed to the actual performing of these experiments and I have to thank Dr Dalyell, Miss Rhodes, Miss Skelton and particularly Mrs Barnes and Dr Delf for permission to use them. A number of them have been published already in various papers on guinea-pig scurvy, particularly those of Dr Delf on cabbage [Delf and Skelton, 1918; Delf and Tozer, 1918], the question of the effect of heat on the growth-promoting properties of cabbage is there discussed but it is not thought out of place to quote the same experiments again here.

SUMMARY:

(1) A large number of dietary experiments on guinea-pigs, originally planned to determine the anti-scorbutic values of various foodstuffs, are analysed and the diets tabulated together with the growth of the animals on the diets.

(2) Growth is found to take place when green cabbage (raw or steamed), green cabbage juice, hay and milk (raw, heated or dried) are added to a diet of oats and bran and water. Orange juice was added when the anti-scorbutic value of the ration was insufficient. Growth is found to vary according to the size of the ration given.

(3) Little or no growth is found to take place when raw white cabbage, white cabbage juice, swede juice, orange juice, onion or germinated peas are added to the diet.

(4) It is argued that the diets could only have been deficient in a dietary factor of the rank of a vitamin and that the growth-promoting factor in these experiments corresponds in its distribution among foodstuffs with the known distribution of vitamin *A*.

(5) As the result of these experiments guinea-pigs are recommended for trial, as suitable for testing the vitamin *A* value of non-fatty foodstuffs, for which rats are for any reason unsuitable.

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XVIII. INVESTIGATION OF THE ANTI-SCORBUTIC VALUE OF FULL CREAM SWEETENED CONDENSED MILK BY EXPERIMENTS WITH MONKEYS.

BY ELEANOR MARGARET HUME.

From the Department of Experimental Pathology, Lister Institute.

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INTRODUCTORY.

EXPERIMENTS have already been made [Barnes and Hume, 1919] on the anti-scorbutic value of raw, dried and heated cow's milk and for some of these experiments monkeys were used. Reference is there made to the literature of the subject, but as far as is known no experimental observations are extant on the anti-scorbutic value of sweetened condensed milk.

It is probable from what is already known of the anti-scorbutic value of milk heated to 100°, or above, that unsweetened condensed milk has lost all or almost all of its anti-scorbutic property, since it is heated at a temperature above 100° in order to sterilise it. With sweetened condensed milk however the high concentration of sugar is depended upon as the preservative after the preliminary pasteurisation of the milk. Concentration is carried out below 55° and is conducted *in vacuo*. It therefore seemed of interest to examine some well-known brand of condensed milk from this point of view and Nestlé's was chosen for the purpose.

In the work already referred to [Barnes and Hume, 1919], the minimum dose of raw milk daily needed to protect monkeys of 2-3 kilos. weight from scurvy was found to be 100-150 cc. daily. It was therefore only necessary to establish the minimum daily dose of condensed milk which would protect the same or similar animals from scurvy, in order to be able to calculate the loss, if any, suffered by the condensed milk.

MATERIAL USED.

The material used consisted of two batches of milk, of which one batch was used in experiment 1 and the other in experiment 2.

By arrangement with the Nestlé Anglo-Swiss Condensed Milk Co., to whom the writer is indebted both for the gift of the material and for all details about it, the milk in each batch was absolutely uniform. Batch 1 was made

on June 7th, 1919, and the experiment was started in August, 1919; batch 2 was made on October 24th, 1919, and the experiment was started in February, 1920. In neither batch was any of the milk from stall fed cows.

The milk is condensed as follows:

Pooled full cream milk is first heated in a vessel with continuous inflow and outflow, the temperature of which is about 80°. It is computed that the milk remains at this temperature for about 3½ minutes. The sugar is next added; during this part of the process there is access of air. It then passes to a vacuum condenser where it boils at about 50° for about three hours and is drawn off when it passes a certain viscosity test. It is then cooled in bulk and during this part of the process there is again access of air.

Figures were provided by the Company giving the degree of condensation, which made it possible to calculate the amount of condensed milk equal to any given quantity of raw milk. These figures differ for every batch.

TECHNIQUE OF THE EXPERIMENTS.

The technique is fully set out in the paper already quoted [Barnes and Hume, 1919].

The basal diet consisted of boiled white rice and wheat germ and the ration of milk was relied on to provide sufficient vitamin *A*.

Both monkeys were male sooty mangabeys (*Cercocebus fuliginosus*) the anti-scorbutic needs of which had been previously ascertained. The one "Rags" had remained in health on a ration of 200 cc. of raw milk daily for 225 days, while the other "Toby" had developed scurvy after 77 days on a ration of 200 cc. dried milk; he was then cured on 200 cc. of raw milk, just scalded, daily. A ration of 200 cc. raw milk daily was therefore more than sufficient to provide anti-scorbutic for either of these monkeys, at a time when they weighed 2-3 kilos. In the course of the present experiments they grew to be monkeys weighing 5 and 4.5 kilos. respectively and were still growing; their anti-scorbutic needs might therefore be supposed to be greater and not less than in the preceding experiment.

EXPERIMENTAL RESULTS.

Experiment 1. This experiment was carried out with the June batch of milk. Both monkeys received the equivalent of 250 cc. of fresh milk daily, Rags for 188 days and Toby for 199 days.

In this period Rags grew from 3370 g. to 4190 g., an increment of 820 g., while Toby grew from 3560 g. to 4320 g., an increase of 760 g. Both remained in perfect health the whole time.

Experiment 2. This experiment was conducted with the October batch of milk. Toby received the equivalent of 200 cc. of fresh milk for 260 days and increased from 4320 to 5020 g., an increase of 700 g. He was in perfect

health at the close of the experiment but the rate of growth was slower than in the previous experiment.

Rags received the equivalent of 150 cc. of fresh milk together with 50 cc. of the same milk, autoclaved at 120° for one hour; this destroyed the anti-scorbutic in the added 50 cc. but made the vitamin *A* value of the ration the same as in the case of Toby. The animal increased in 246 days from 4190 to 4570 g. an increase of only 380 g., less than half the growth in the former shorter period, and not much more than half the growth shown by Toby on the same vitamin *A* ration. The two animals' rates of growth in the first experiment on identical rations were almost exactly the same: the animal however appeared to be in perfect health at the close of the experiment.

The lag in Rags' growth on the 150 cc. ration might be due to a slight anti-scorbutic insufficiency, but since in previous experiments [Barnes and Hume 1919], the minimum requirement for monkeys of 2 to 3 kilos. was found to be 100 to 150 cc. of raw winter milk a day, it cannot be definitely stated that any loss of anti-scorbutic potency is demonstrated in the condensed milk.

The loss, if any, is small and the same is also true with regard to vitamin *A*. The present experiments were not made with a view to testing this property, but save for the wheat germ, the milk provided the sole source of vitamin *A* in the diet. Previous experience [Barnes and Hume, 1919] has shown that about 200 cc. of autoclaved milk daily provide a supply of vitamin *A* which enables good growth in monkeys of 2-3 kilos. to take place.

A recent experiment of Hopkins [1920] has shown that the vitamin *A* value of butter is only destroyed on heating at 120° for four hours, when access of air is permitted. Recent experiments by Delf [1920, p. 227] suggest that the anti-scorbutic vitamin is also much more susceptible to heat when free access of air is permitted. Unpublished experiments by Zilva show that there is loss of anti-scorbutic potency in substances exposed to the action of ozone. Doubtless these observations give the explanation of the non-destruction of vitamins in the condensation of Nestlé's sweetened condensed milk, for though the milk is heated for a very considerable time, though at a low temperature, it is heated *in vacuo*.

One important point should however be made with reference to the use of sweetened condensed milks in infant feeding. The above experiments show that there is little if any vitamin destruction in such milk but it must be borne in mind that in the experiments the milk was reconstituted to have as nearly as possible the same fat and vitamin (assuming no destruction) content as raw milk. That is to say that it was diluted at most four times (one vol. condensed milk to about three vols. water) whereas instructions for the use of the milk for infants suggest a dilution of at the lowest eight times and at the highest very much more. So large an addition of water produces a dilution of the fat and vitamins to an extent likely to be dangerous, although the high

percentage of sugar gives an adequate calorie supply, a danger to which attention was drawn by Coutts [1911].

Thanks are due to Dr C. J. Martin and to Dr H. Chick for help in directing the experiment and to Miss H. Henderson Smith for help in caring for the monkeys.

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OBSERVATIONS ON CASES OF RICKETS IN AN OUT-PATIENT DEPARTMENT.

BY

HELEN M. M. MACKAY, M.D.LOND., M.R.C.P.,

BEIT MEMORIAL RESEARCH FELLOW; ASSISTANT PHYSICIAN, QUEEN'S
HOSPITAL FOR CHILDREN.

AT the present time, in spite of the large amount of attention which has been focussed upon the etiology of rickets, there appears to be no general agreement as to its cause. Mellanby believes that the ultimate factor determining the occurrence of rickets in puppies of a susceptible age is the absence in the diet of a sufficient quantity of "anti-rachitic factor"¹—a substance which has been held to be identical with fat-soluble A.² Yet Noël Paton and his co-workers, who, in the course of their investigations on rickets, have carried out somewhat similar experiments in dogs, consider that "some other factor than diet is the prime cause of rickets,"³ and that rickets "is directly correlated with overcrowding and confinement in insanitary houses."⁴ Hess, who, like most clinical workers, holds that rickets "is primarily a dietetic disorder," believes that cod-liver oil "is almost a specific" as a prophylactic or curative remedy, but at the same time holds that fat-soluble A is only one among several factors concerned in the pathogenesis of rickets,⁵ and that probably "the danger to infants of a diet deficient in fat-soluble vitamines is slight, provided it includes sufficient calories and is otherwise complete."⁶

Bull, commenting on his clinical and experimental investigations on rickets among foxhound puppies, concludes that "although the faulty nature of the food was closely associated with the production of the disease, it has been shown that it was not capable of producing the disease when other conditions were altered," and that rickets is probably "due to a reaction on the part of the organism to an infection or chronic intoxication."⁷ Erdheim (working in Vienna during the time of shortage and of high prices, when very many among the population were under-nourished) examined pathologically a large number of caged rats which had spontaneously developed rickets at this time. He gives in his published report no

particulars of the diet of these animals, but he shows a close relationship to exist between hypertrophic changes in the parathyroids and the presence of rachitic changes in the rats' bones—those rats having rickets had enlarged parathyroids with microscopic appearances indicative of hyperactivity of the glands.⁸ Erdheim also removed the parathyroids in rats, and bone changes similar to rickets followed this operation. This apparent contradiction he explains on the theory that the parathyroids in some way control calcium metabolism, and that in spontaneous rickets the hypertrophy indicates an effort at compensation on the part of the organism. Erdheim's observations are of considerable interest, in view of the following facts: (a) The occurrence of a condition resembling tetany as a result of the experimental removal of the parathyroids;⁹ (b) the extremely frequent association of tetany with rickets and artificial feeding in children; and (c) the fact, as stated for instance by Holt, that in children "the active symptoms of tetany may be controlled by calcium,"¹⁰ the metabolism of which is disturbed in rickets. These facts would suggest that the hypertrophy of the parathyroids may, as Erdheim suggests, be a protective measure against some common cause giving rise to abnormal calcium metabolism, to an increase of guanidin in the blood, and in some way to both rickets and tetany.

Guanidin, a product of decomposition of protein, is, according to Noël Paton, the chemical substance which gives rise to the symptoms of tetany.¹¹ Morpurgo¹² isolated from the organs of white rats which suffered from a spontaneous outbreak of osteomalacia in his laboratory a Gram-negative diplococcus, and succeeded in producing the disease in hundreds of other rats by subcutaneous inoculation of either the pure culture or the organs of animals containing the diplococcus. When adult animals were the subject of experiment a disease resembling osteomalacia was produced, but with newborn rats the effects were closely similar to the changes observed in rickets in children. Schmorl, who examined Morpurgo's specimens, agrees that the histological changes are identical with those of rickets.

Looser¹³ is of opinion that rickets and osteomalacia are one disease, and supports his views by the results of clinical, radiographic, and pathological examinations of cases coming under his observation during a period of many years. He believes that the cause is most probably an infective one.

The papers heretofore quoted are all recent publications, but Bland-Sutton, working on animals at the Zoological Gardens, described over forty-five years ago how gerbills in captivity developed a condition either of rickets or of osteomalacia according to their age, and concluded that the causes of these two conditions are one and the same.¹⁴ He showed, too, how monkeys frequently developed rickets, and he considers the "rickets of maturity" in monkeys comparable with osteomalacia.¹⁵ His experiments on lion cubs at the Zoo, and the prevention among them of rickets

by means of dietetic measures,¹⁶ are well known, and his observations indicate that both osteomalacia and rickets are mainly dietetic diseases, and can be prevented by an adequate diet.

Thus it will be seen that there are at present in our possession no facts conclusively proving the cause or causes of rickets, though it would appear to be at least in part a dietetic disease, and that its occurrence in rats is associated with changes in the parathyroid glands.

INVESTIGATION OF 51 RACHITIC OUT-PATIENTS.

In 1920 I carried out some clinical investigations on rickets among children attending the out-patient department of the Queen's Hospital for Children and the infant clinic at University College Hospital. In all I collected 51 cases of rickets in children between 5 months and 4 years of age, of whom 46 were under 3 years of age. I limited myself to cases in which the condition was, so far as I could tell, still active. The majority were heretofore untreated, and a few were cases growing worse, in spite of previous treatment.

I began with the belief that rickets was mainly, perhaps entirely, a dietetic disease, and it seemed reasonable to start from the hypothesis that it is due, in part at least, to a want of fat-soluble A and to investigate the cases from this point of view.

My results go to prove the impossibility of obtaining data of sufficient accuracy from out-patient work to give any conclusive results. There was a very wide margin of error in the histories given me; irregular attendances, intercurrent diseases, and radical changes made by the mother in the food (which, perhaps, in spite of careful questioning, I only discovered by chance weeks later), also invalidated very many of my records. These sources of error point to the absolute necessity that an investigation in clinical work undertaken with a view to elucidating the cause of a supposed dietetic disorder should be institutional. Nevertheless, some points of interest appear worth recording.

The Diet of Rachitic Cases.

The history revealed that in 35 out of 51 rachitic children (that is, nearly 69 per cent.) artificial feeding was begun before six months of age. Allowing for the uncertainties of the history, there seems no doubt that in at least 29 of these 35 cases the diet would have been markedly deficient in fat, as compared with human milk, for a period of anything between one and a half to seven and a half months before the age of 8 months. The degree of deficiency it was quite impossible to estimate. But, as against this, it must of course be remembered that, unless fed on a dried milk (which is very usually given in a strength roughly equivalent to undiluted cow's milk), the vast majority of artificially-fed infants of the poorer classes in London receive a diet containing a considerably smaller

percentage of fat than human milk, and I think there can be no question that the majority of these do not develop rickets.

Twenty-two of the 35 bottle-fed children, and probably a considerably larger number, received for a period of six weeks or more before the age of 6 months a food containing a large percentage of carbohydrate—for example, sweetened condensed milk, or a food with added starch. The older children on mixed diets appeared to receive a large quantity of carbohydrate food, but I did not come to any conclusion as to whether it was more than that consumed by the majority of children of corresponding age of the same social class.

In at least two children under nine months of age rickets developed on a diet of breast-milk only, and it is interesting to note the particulars obtained of the diet of one of the two mothers in question (the husband was a casual labourer at the docks, and sometimes only able to get one day's work in the week):

Breakfast: A cup of tea with milk.

Dinner: Potatoes daily, suet or batter pudding (about four days a week), or meat or fish (about three days a week), greens once or twice in the week, bread sometimes.

Tea or Supper: Generally none, occasionally bread and margarine.

It seems that under these circumstances the milk secreted might well have been poor in quality (*cf.* Dalyell, BRITISH MEDICAL JOURNAL, July 31st, 1920). Unfortunately it was not possible to obtain a specimen for examination. In the second case I was not able to get details of the mother's diet.

A communication made to me by a medical woman working in India has some bearing on the association of rickets with diet. She informed me that, except among the "mission" children, she rarely saw a case of rickets in a native. Among the "mission" children it was not uncommon. She attributed this to the fact that many of these children were bottle-fed, whereas a child reared on the bottle was comparatively rare among the general population.

Antiscorbutic in the Diet.

I was interested in the question of whether an antiscorbutic deficiency could play any part in the production of rickets. It is obvious that if fat-soluble A is often deficient in an infant's food as a result, for instance, of dilution, the proportion of the less stable antiscorbutic vitamine may be correspondingly even further reduced in the same food, and even fresh cow's milk is not rich in antiscorbutic. Hess and Unger calculate that an infant probably requires the antiscorbutic equivalent of 1 pint of fresh cow's milk per day.¹⁷ It was impossible to ascertain from the data available whether there was any antiscorbutic deficiency in the diets as a whole. However, in view of Hess's suggestion that a condition of subacute

scurvy is not infrequently mistaken for rickets,¹⁸ it is of some interest to note the following two cases which were sent to me as rickets: Rose M., aged 9 months, had been fed on proprietary foods since 2 weeks old, but had had "an orange quarter to suck twice a day since 6 months old." She was brought to the hospital because "she screamed when the ankles were touched." When seen there was beading of the ribs, and enlargement of the epiphyses of the wrists and ankles, the limbs appeared very tender, the gums were swollen and bluish-red round the erupted teeth, and the capillary resistance test of Hess and Unger¹⁹ was strongly positive, a test which, though probably not pathognomonic, yet I think clinches the diagnosis of scurvy in this case. Unfortunately this child was seen once only.

The directions given by these authors for the test are briefly as follows: The blood-pressure band of the sphygmomanometer—Tycos apparatus was used by them—is put round the arm and the pressure raised and kept at, say, 90 mm. of mercury for three minutes. The object is to obliterate the flow of blood and so produce cyanosis without obliterating the pulse. They claim that, after removal of the band, infantile scurvy cases show very numerous points of bleeding, normal cases none, or perhaps one or two—petechiae just below the pressure band are to be ignored. Obviously there is more probability of obtaining petechial haemorrhages, other conditions being equal, in a child which has raised its blood pressure by crying than in one which is quiet—a point, I think, to be borne in mind in doubtful cases.

The second child, Bertram S., aged 1 year 3½ months, would have appeared from his history to be receiving a sufficiency of antiscorbutic, but the teeth were decayed, the mouth in a condition of stomatitis, the capillary resistance test positive, and in sixteen weeks he had lost ½ lb. in weight. Therefore he was put on large doses of orange juice (about 3ij daily), besides local treatment for the mouth, and he rapidly improved, and gained an average of over 8 oz. a week for five weeks—facts which suggest that the condition may have been subacute scurvy. This child also showed beading of the ribs and enlargement of the epiphyses at the wrists as well as tuberculous dactylitis. I presume that clinically the enlargement of the epiphyses in both these cases would be held to justify a diagnosis of rickets whatever other conditions were also present.

Result of Treatment with Single Additions to the Diet.

I pass now to the results of treatment. I divided the cases as I saw them into five groups, endeavouring to keep all the groups as equal as possible with regard to age and types of case composing them. To the previous diet of the children of each of these groups I made one of the following additions:

1. An animal fat presumably rich in fat-soluble A, namely, cod-liver oil 3 ij daily.

2. An animal fat also presumably rich in fat-soluble A, namely, butter 3 ij daily.

3. An animal fat *plus* a substance rich in antiscorbutic, namely, butter 3 ij *plus* orange juice 3 iij daily.

4. An animal fat *plus* a substance rich in water-soluble B, namely, butter 3 ij *plus* marmite 3 ss daily.

5. A vegetable oil presumably much lower in fat-soluble A than the foregoing animal fats—namely, cottonseed oil 3 ij daily.

The fats were given as emulsions, and all the substances, including the orange juice, were dispensed at the hospital. Of course any outstanding symptoms, such as constipation, diarrhoea, or cough, had to be treated, but I avoided, as far as possible, giving the mother any directions as to feeding or hygiene in the home, with a view to keeping those factors unaltered. The results were disappointing. Owing to the small number of cases which attended with any regularity I have grouped under the one head of "butter" in the following table all the cases receiving butter, butter and orange juice, or butter and marmite.

Period March to July, 1920. Results of Treatment.

	Addition to Diet.		
	Butter 3 ij daily.	Cod-liver oil 3 ij daily.	Cottonseed oil 3 ij daily
Number of cases	9	4	6
Average age	25½ mos.	18¾ mos.	21¾ mos.
Average deficiency of weight (compared to normal child) at beginning of treatment	75 oz.	40 oz.	71 oz.
Average deficiency of weight at end of treatment	79 oz.	32½ oz.	59 oz.
Total period of treatment of all cases	58 wks.	34 wks.	45 wks.
Average duration of treatment for each case	6½ wks.	7½ wks.	8½ wks.
Average change in deficiency of weight	Deficiency increased by 4 oz. 2 oz. nearly.	Deficiency decreased by 7½ oz. 2½ oz.	Deficiency decreased by 12 oz. 2½ oz.
Average weekly gain of each child			

Obviously the numbers are extremely small, and to my knowledge the general diet was modified in several cases during the period of observation, so that I do not think that any deductions can be drawn from differences between the groups. It, however, is noteworthy that no group showed any striking improvement. Some of the cases included, however, had only two consecutive weeks of treatment. About half of the 19 cases showed definite improvement in their general condition, but none could be said to show any definite improvement in the bone lesions, and the sweating persisted in those cases in which it had been present at the beginning of the period. These observations extended from March to the middle of July.

Result of More Extensive Changes in the Diet.

In July I decided to see if I could obtain any marked improvement in the children by attempting to bring about a greater change in the diet. I gave the mothers instructions as to the general feeding, particularly concerning any obvious errors in the dietary, usually instructing them to increase the quantity of animal fat and protein and cut down the quantity of carbohydrate in the diet. At the same time I endeavoured to push the quantity of cod-liver oil—in some cases I gave as much as 3 vj daily to a child of about one year. The improvement in this set of cases as compared with the last set of 19 was marked. (This group includes many of the cases already treated in the first group.)

Table showing Results following General Directions as to Diet, with increasing doses of Cod-liver Oil (3 ij to 3vj daily).

Number of cases	18
Average age (in months)	23
Average deficiency of weight at beginning of treatment	57½ oz.
Average deficiency of weight at end of treatment	41 oz.
Total period of treatment of all cases	115 weeks
Average duration of treatment	6½ weeks
Average change in deficiency of weight:	Deficiency decreased by 16½ oz.
Average weekly gain	4 oz.

Not only did the weight approach the normal, but in nearly every case there was a definite improvement in the child's general condition, and in several cases there

was an improvement in the bone lesions—for example, less marked beading of the ribs or reduction in size, of enlarged epiphyses; 9 of the 18 cases had excessive sweating at the beginning of the period, and in 5 of these the condition improved or disappeared. On the other hand, David H., aged 1 year and 3 months,

for example, who showed very marked improvement in weight and general condition (his weight increased from 11 lb. 14 oz. to 15 lb. 4 oz. in eight weeks), showed on

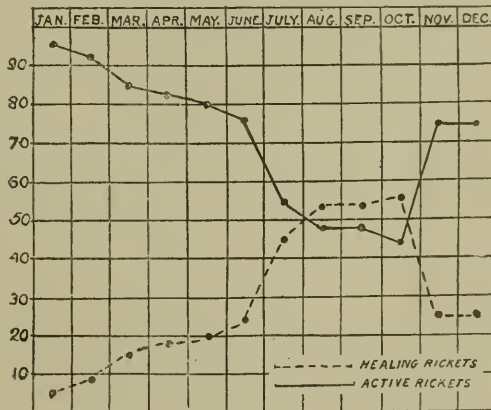
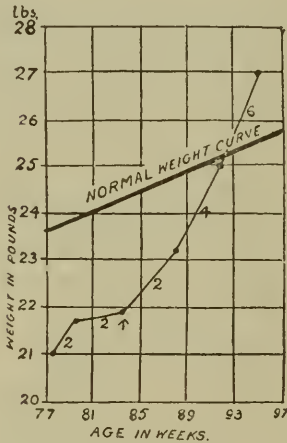


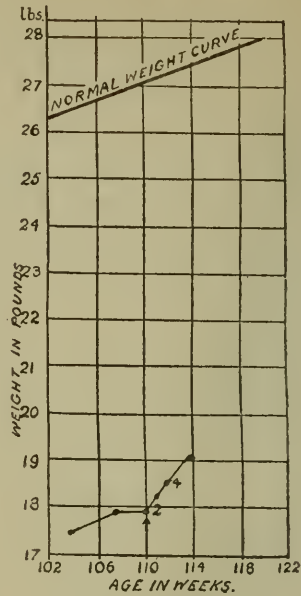
Chart showing percentage of cases examined *post mortem* (386 in all) with active and healing rickets for each month of the year. Compiled after Schmorl. Reproduced from "A Study of Social and Economic Factors in the Causation of Rickets." by M. Ferguson and L. Findlay (Medical Research Committee Report).

improvement in the sweating. This child at the end of the period was receiving 3vj of cod-liver oil daily, and I presume the sweating was evidence that the rachitic condition was still active in spite of the large consumption of cod-liver oil.

In connexion with this group of cases it is of interest to



Victor B.



Elsie B.

Weight charts of two cases in which there was definite improvement from the date at which general dietetic treatment was begun. The arrow indicates the date at which directions as to general diet were given; the numbers against the weight curve indicate the daily dose of cod-liver oil in drachms.

Victor B., aged $1\frac{1}{2}$ years, was the son of a pastrycook, and received large quantities of "pastries." He has attended for seventeen weeks, and shows improvement in the size of the rosary and wrist epiphyses.

Elsie B., aged 2 years, is the child of a widow in extremely poor circumstances.

note the seasonal curve of rickets reproduced herewith. It will be seen that July shows a sharp rise in the incidence of "healing rickets." Whether the contrast between this group of 18 cases as compared with the previous group is merely due to the season at which treatment was carried out, or whether the dietetic treatment was an important factor, it is impossible to say with certainty, but from the observations of Dalyell²¹ on the effect of small additions of animal fat to the diets of children suffering from arrested development in Vienna, my impression is that the general dietetic treatment played an important part in the improvement which occurred. (See weight charts.)

Summary.

My observations on these cases are, therefore, so far as they go, in accordance with the fairly general belief that rickets is a dietetic disease and commonly develops in children receiving insufficient fat and too much carbohydrate in the food, and that it shows itself most commonly among those who have been bottle-fed. In the case

of one child known to have developed rickets while at the breast it is evident that the mother was receiving an inadequate diet, and it is probable that the infant's diet was also inadequate as a result.

The number of cases observed was unfortunately small, but they gave no indication that fat in the form of cod-liver oil, butter, or cottonseed oil exerts any specific curative influence on the disease in doses of ʒij daily. On the other hand, there was a marked improvement in a group of 18 cases treated during July, August, and September with larger doses of cod-liver oil, together with advice as to diet, but it is certain that this improvement was in part due to the season of the year.

Still has drawn attention to the fact that children suffering from coeliac disease in which there is failure to absorb fat "show a remarkable freedom from rickets."²⁰ In two experiments recently carried out at the Lister Institute, kittens given a diet deficient in fat-soluble A developed a condition showing a striking resemblance to that of children with coeliac disease: together with arrest of growth and distension of the abdomen there was muscular feebleness and apathy with a "pathetic interest in food." A small liver appears in many cases to be a feature of the disease. All these are points to which Still draws attention in coeliac disease in children.²² These facts would suggest the conclusion that a deficient absorption of fat-soluble A accounts for the picture in coeliac disease. If that be so, some other factor and not a deficiency of fat-soluble A alone must be concerned in the production of rickets.

In conclusion, I should like to express my great indebtedness to Dr. C. J. Martin for his help and for his kindness in arranging that the expense of this investigation should be defrayed by the Lister Institute; to Dr. E. A. Barton, who took much trouble in collecting suitable cases for me at his infant consultations at University College Hospital; to Miss S. E. Jacob, pharmacist to the Queen's Hospital, and to the many others who were kind enough to send me cases and to co-operate in other ways.

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III. THE EFFECT ON KITTENS OF A DIET DEFICIENT IN ANIMAL FAT.

BY HELEN MARION MACPHERSON MACKAY.

Beit Memorial Research Fellow.

The Department of Experimental Pathology, Lister Institute.

(Received November 15th, 1920.)

Mellanby [1919] in the course of his experiments on dogs has come to the conclusion that rickets is a dietetic disease, and that the ultimate cause is a deficiency in the diet of "anti-rachitic factor" a substance which he thinks is probably identical with fat-soluble *A*. He has said that 100 % of young puppies fed on his basal diets develop rickets.

Since the cat, like the dog and man, subsists on a mixed dietary it was decided to investigate whether kittens fed on a diet similar to Mellanby's basal diet would develop rickets. Before describing the experiments on kittens it is of interest to compare the normal diet of young pups and kittens [Tibbles, 1912]:

	Cat's milk	Bitch's milk	Cow's milk
	%	%	%
Protein	9.12	11.15	3.57
Fat	3.33	9.57	3.69
Sugar	4.91	3.08	4.88
Ash	0.61	0.73	0.71

It will be seen from the foregoing table that the percentage of protein is far higher in the milk of the cat and the bitch than in that of the cow; bitch's milk contains nearly three times the percentage of fat present in cat's milk, and the fat content of cat's milk and cow's milk is roughly the same. If the percentage of fat in these three milks could be taken as an index of the quantity of fat-soluble *A* present it would point to the fact that the puppy requires a large quantity of this in its diet, and would afford an explanation on Mellanby's hypothesis of the apparently great liability of young dogs to rickets as compared with other domestic animals.

The latest of Mellanby's published basal diets consists of the following substances:

Separated milk	...	250-350 cc. daily
White bread...	...	<i>ad lib.</i>
Linseed oil	...	5-15 cc. "
Yeast	...	5-15 g. "
Orange juice...	...	3 cc. "
Sodium chloride	..	1-2 g. "

All the fat-soluble *A* content of milk is not present in the fat [see McCollum, 1917], but no accurate data are at present available from which it is possible to make any trustworthy calculation of the amount of this factor present in the quantity of separated milk (250–350 cc.) given in the foregoing diet.

EXPERIMENT 1.

Housing and Diet of Kittens.

The five kittens used in the experiment belonged to three litters. They were housed and fed in two pens (controls in one and experimental animals in the other) in a warmed room. They were allowed out for several hours every day and had abundant exercise. The diet is given in the following table:

Control kittens, Nos. 2 and 3					Experimental kittens, Nos. 1, 4, and 5
Whole milk <i>ad lib.</i>	Machine skimmed milk with 3 % olive oil <i>ad lib.</i>
White bread <i>ad lib.</i>	} Same as for controls
Orange juice 1 cc. (hand fed) alternate days				...	
Marmite 2 g. given in milk, whole not consumed					

The milk, whole and skimmed, was brought to the boil and allowed to cool before being given to the kittens—a proceeding which probably destroys only a very small part of its anti-scorbutic value [Barnes and Hume, 1919]. The average fat content of a week's supply of machine skimmed milk estimated by Soxhlet's method was 0.15 %¹. The olive oil used after the first six weeks was kindly supplied by Dr Zilva from a consignment of autoclaved oil which gave a negative result for fat-soluble *A* in rat experiments (linseed oil had been tried but was refused by the kittens). The oil emulsified fairly well with hot skimmed milk but separated out when allowed to stand, so that the quantity of oil consumed was not necessarily 3 % of the skimmed milk intake. The dose of orange juice was a small one, but it was thought that with a diet consisting chiefly of milk a small dose would ensure against anti-scorbutic deficiency. A larger amount was at first given but appeared to give rise to diarrhoea.

The marmite, which was used in place of the yeast to supply additional water-soluble *B* accessory food factor, was given in the day's supply of milk. It is not possible to give the food intake of each individual kitten as the food (with the exception of the orange juice which was given with a pipette) was left in the pens. But the following table gives the average consumption; the quantity of bread is a very rough approximation as it was impossible to estimate with accuracy the residues left.

¹ On a few occasions dried machine skimmed milk or hand skimmed milk was given on account of the failure of the supply of separated milk from the dairy.

Average daily food consumption of each kitten of experiment 1.

				Control kittens 2 and 3	Experimental kittens 1, 4, and 5
Whole milk	About 200 cc. each	—
Skimmed milk with 3 % olive oil				—	About 145 cc. each
White bread	„ 25 g.	„ 20 g.
Orange juice	0.5 cc.	0.5 cc.
Marmite	1-2 g.	1-2 g.

Course of experiment.

The accompanying charts (Figs. 1 and 2) show the weight curves of the kittens. But at this stage it must be mentioned that every one of the five kittens, especially the three experimental animals, was latterly very heavily infested with fleas, and that there were in each case at post mortem vast numbers of tape worms in the small intestines. This tape worm, the *Dipylidium caninum*, has for its secondary host the dog or cat flea. To what extent these parasites affected the ultimate result is not clear, but all the kittens suffered from time to time with loose stools, their rest was disturbed by the fleas, and at the end of the experiment neither of the two control animals was in perfect condition; kitten 2 was well nourished and active but had loose stools, No. 3 was very thin. The experimental kittens were from the first more keenly interested in their food than the controls—they appeared to feel more hungry though their consumption of food was on the average smaller. About the sixth week of the experiment it was obvious that the experimental kittens were thinner than control kitten No. 2 which showed a steady gain in weight after the first three days (control No. 3 was however thin also). By the tenth or eleventh week the experimental animals were extremely emaciated, with coats in poor condition and were becoming less active. The emaciation, the condition of the coat and the inactivity became steadily worse and latterly the abdomens were much distended. At the end of the experiment there was an obvious difference in size between the two control kittens and the other two of the same litters. The difference in length from tip of nose to end of tail was 2 ins. and 1 in. respectively.

All the kittens appeared anaemic, possibly partly as a result of the tape worm infection. The blood count for kitten 5 (on the deficient diet) on the 114th day of the experiment is given below:

		Normal blood count for cat [Gulland and Goodall, 1914]
	Kitten 5	
Red blood corpuscles	4,390,000 per cmm.	8,000,000 per cmm.
Haemoglobin ...	21 %	70 %
White blood corpuscles	22,300 per cmm.	18,000 per cmm.

One of the skimmed milk animals, kitten 1, died on the 82nd day, the other two, kittens 4 and 5, were killed on the 114th day.

Post mortem findings.

At post mortem no evidence of rickets was discovered. The pathological findings are given below. It will be remembered that the three kittens on the deficient diet were kittens 1, 4 and 5, those on the theoretically complete diet kittens 2 and 3.

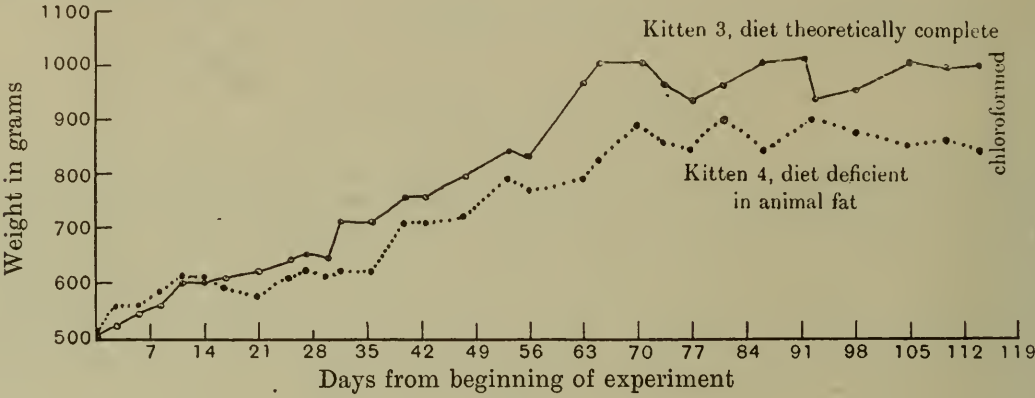


Fig. 1.

Control Kitten, 3 ♀ o—o } Same litter (6 weeks old)
Experimental Kitten, 4 ♀ ••••• }

It will be seen that this Control Kitten did not thrive

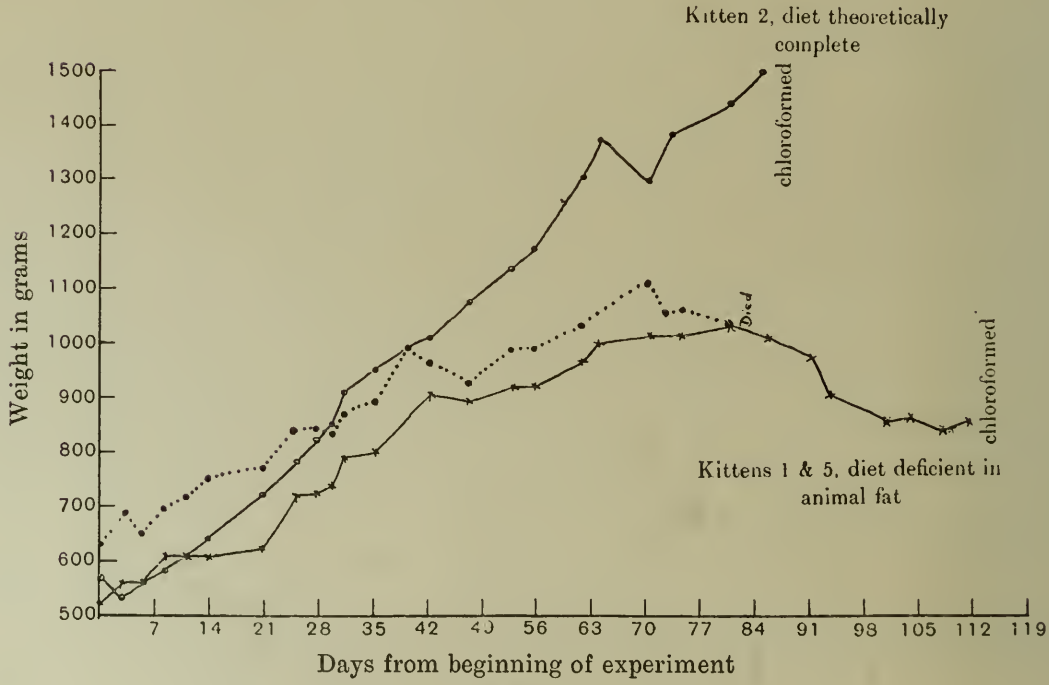


Fig. 2.

Control Kitten, 2 ♀ o—o } Same litter
Experimental Kitten, 1 ♀ ••••• }
Experimental Kitten, 5 ♀ x—x }

EXPERIMENTAL KITTENS 4 AND 5. Killed 114th day. Extremely emaciated. *Costo-chondral junctions* slightly enlarged. The histological changes as will be seen from the following paper written by Miss F. M. Tozer [1921] resembled those found in guinea-pigs and rats receiving a diet deficient in fat-soluble A.

Stomach and intestines were distended with more or less complete loss of the normal puckering. The walls were much thinner than in the normal animals. The changes were similar to those described by McCarrison [1920] in animals receiving deficient diets. *Liver* pale and fatty looking—that of kitten 5 small compared to the controls. *Kidneys* very pale. *Thymus* absent.

EXPERIMENTAL KITTEN 1. Died 82nd day. Extremely emaciated.

Costo-chondral junctions very slightly enlarged [see Tozer, 1921]. *Stomach and intestines* appeared normal. *Liver* pale fatty and small. *Kidneys* appeared normal. *Thymus* absent.

CONTROL KITTEN 2. General condition excellent, nothing abnormal discovered excepting the parasites which were found in all the animals.

CONTROL KITTEN 3. Very thin. No cause found unless the condition was due to the presence of the parasites.

The teeth of all the kittens were perfect. In the two control animals whose costo-chondral junctions were normal [but see Tozer, 1921] the thymus gland was present, whereas as will be seen above it was absent from the three experimental animals.

Summary. To summarise it appears that the chief effects of the diet deficient in fat-soluble *A* on these three six-week-old kittens were emaciation, arrest of growth, abdominal distention with (in two animals) atrophy of the walls of the stomach and intestines, and changes in the costo-chondral junctions similar to those seen in guinea-pigs and rats suffering from deficiency of the same accessory food factor.

The weak point of the experiment is of course the presence of the intestinal parasites and the fact that one of the two controls did not increase satisfactorily in weight.

EXPERIMENT 2.

Children who develop rickets generally do so during the period of the first dentition—a stage of development at which they are, comparatively, much less mature than six-week-old kittens. Therefore while the first experiment was running it was decided to start a second one, this time with younger animals.

Age and diet of kittens. Of a litter of four kittens aged 16 days, three, after certain preliminary “test feeds,” were removed from the mother and hand fed entirely until nearly five weeks old, and partially until 7–8 weeks old. They were fed three-hourly from 10 a.m. to 10 p.m. with warmed food from a pipette or baby’s bottle until old enough to feed themselves. The food was on the same lines as in the last experiment.

Average daily food consumption of each kitten of experiment 2.

	Control kitten 9	Experimental kitten 6	Experimental kitten 7
Whole milk	About 180 cc.	—	—
Skimmed milk with 3 % olive oil ...	—	About 145 cc.	About 160 cc.
White bread supplied <i>ad lib.</i> after 3rd week of experiment	A very small quantity	} Same as for control	
Orange juice hand fed after 3rd week	0.5 cc.		
Marmite	1–2 g.		

Course of experiment (see Fig. 3).

The two experimental kittens appeared always ravenous, though latterly their food intake was decidedly smaller than that of the control. This was a striking feature almost to the time of death. About the 4th day kitten 7 had diarrhoea, a condition from which both experimental kittens frequently suffered, especially kitten 7 (though both had masses of hard faeces in the large intestine at death). By the 8th day kitten 7 was less active than the control, the abdomen was distended and it walked very unsteadily on a broad base with the back bowed. By the 12th day kitten 6 was in much the same condition. They became progressively worse in all respects, the coat was matted and dirty and swarming with fleas, the hair coming out and there

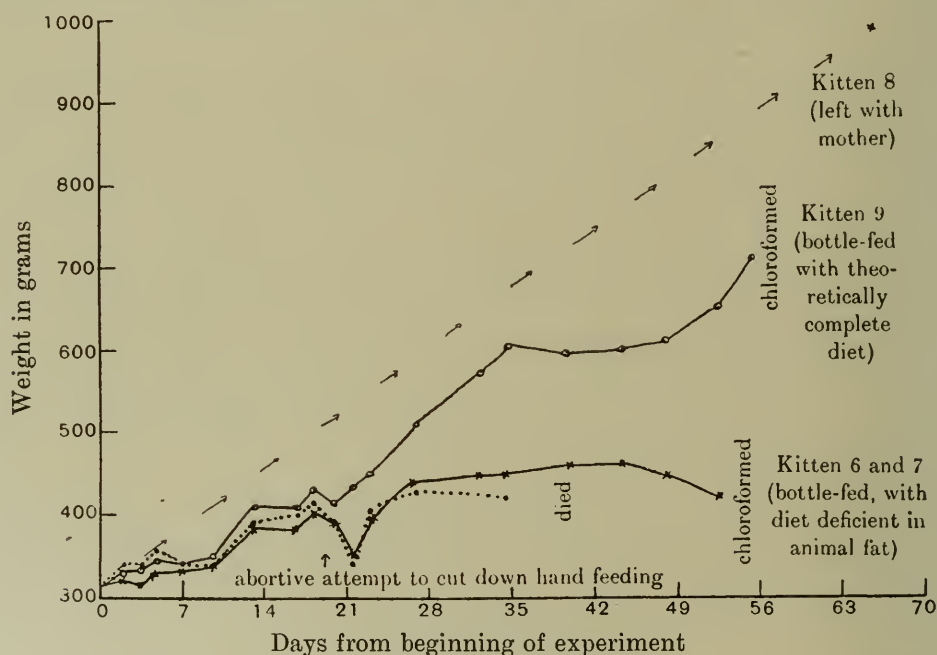


Fig. 3.

Control Kitten, 9 ♀ o—o
 Control Kitten, 8. Left with mother, final weight indicated → x
 Experimental Kitten, 6 ♂ ×—×
 Experimental Kitten, 7 ♂•

was emaciation and arrest of growth. By the end of the experiment the contrast was striking. Kitten 9, though considerably smaller than the single kitten left with the mother, was a healthy active young cat, whereas the two animals on deficient diets were wasted dwarfs with ballooned abdomens and matted hair. They presented a miserable spectacle. The lengths of the kittens quoted below give no idea of the apparent difference in size. Kitten 7 died on the 38th day with an attack of vomiting, kitten 6 appeared moribund and was chloroformed on the 54th day.

Kitten	Comparative lengths of kittens (inches)	
	35th day	54th day
9	14.75	17.75
7	12.5	—
6	13.0	14.75

Post mortem findings.

At post mortem it was found that all three kittens had tape worms, the control far more than the two on the deficient diet. The fat and the healthy looking well-developed muscles of control kitten 9 were in marked contrast to the pale flabby wasted tissues of the two small experimental kittens. The two experimental animals had slightly enlarged costo-chondral junctions (for the histological changes, which resembled those found in the earlier experiment, see Tozer [1921]). The rib junctions of the control were normal. The teeth were perfect.

The *stomach and intestines* showed dilatation and atrophy of the wall. The *liver* was pale and small compared to that of kitten 9. The *kidneys* appeared normal. The *thymus* was absent from all three kittens.

Summary. The results of this experiment were similar to those of the last, but the arrest of growth, emaciation, abdominal distention, weakness and the hankering for food were early and very marked features. The picture seems extremely like that presented by children suffering from coeliac disease, a condition in which there is failure to digest fat, arrest of growth, emaciation, distention of the abdomen and hankering for food, as well as, in some cases, a diminution in size of the liver. Still [1918], after remarking on the apathy and langour of children with coeliac disease, observes "There is one subject in which almost all children with coeliac disease show a pathetic interest—namely their food; often indeed it is their one subject of conversation." He however attributes this to the meagre dietary which has formed a part of their treatment.

Criticisms of the experimental procedure.

If these experiments were repeated it would be desirable to feed the kittens separately in order to ascertain the food intake of each, and for the same reason to give the oil by hand instead of as an emulsion in the milk.

A better control would be provided by feeding the whole litter on skimmed milk, and to the control animals giving say cod liver oil, which is rich in fat-soluble *A*, in place of the olive oil of the experimental animals, for it is probable that the mechanical factor introduced by the oil was in part the cause of the diarrhoea in kittens 6 and 7. It might be as well also to give a larger dose of anti-scorbutic, possibly in the form of neutralised orange juice.

As already pointed out the presence of the tape worms and their hosts the fleas introduced an unlooked-for difficulty.

EXPERIMENT 3.

Young rats aged 13 days (the eyes had opened on the 12th day) were put on a diet of white bread, skimmed milk with 3 % olive oil and small pieces of orange pulp. They consumed a much smaller quantity of milk as compared

with bread than did the kittens—but as will be seen from the weight chart (Fig. 4) their weight was not greatly below normal at the end of 122 days—their general condition was excellent. It would appear therefore that kittens require a larger proportion of fat-soluble *A* in their dietary than do young rats.

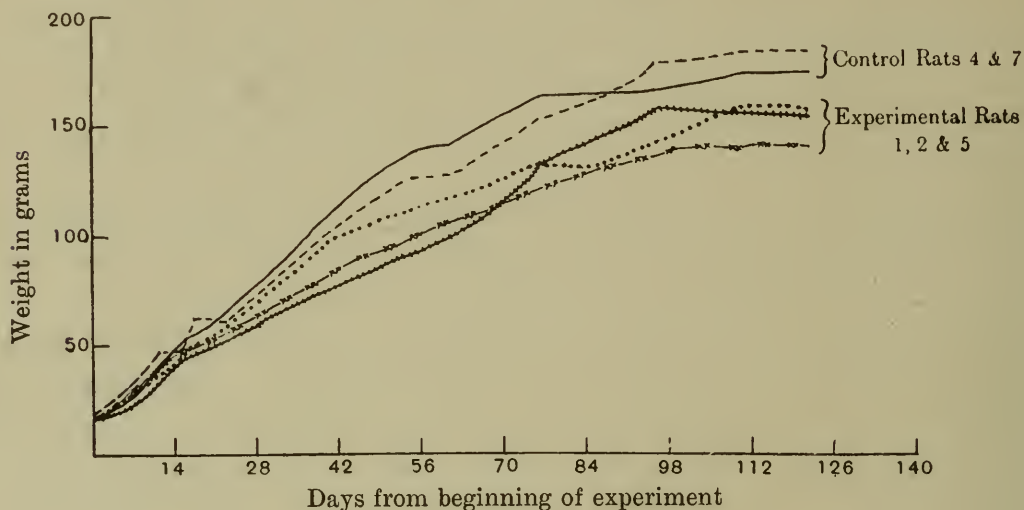


Fig. 4.

Control rat, 4 ♀	——	Whole milk, bread and orange juice from 13 days old
„ rat, 7 ♀	- - - -	
Experimental rat, 1 ♀	× × — × ×	Skimmed milk with 3 % olive oil, bread and orange juice from 13 days old
„ rat, 2 ♀	+++	
„ rat, 5 ♀	

GENERAL SUMMARY AND CONCLUSIONS.

In the experiments described above it was found that the six-week-old kittens when fed on a diet deficient in fat-soluble *A*, but otherwise theoretically adequate, became emaciated and finally ceased growing. They suffered from abdominal distention and diarrhoea; at post mortem it was found that the walls of the intestine were thin, and there were changes in the costo-chondral junctions resembling those found in young guinea-pigs and rats suffering from deficiency of the same accessory food factor. The thymus was present in two control animals and absent from the three experimental animals, but in the next experiment was absent from control and experimental animals alike. The food of these experimental kittens consisted of skimmed milk, olive oil and bread, with marmite and orange juice. Some weak points in the experiment have been pointed out.

Analogous changes were observed in a second experiment on kittens aged 16 days, which were hand-fed, and given a similar dietary—but in this case all the changes were much more rapid and striking. The appearance presented by these animals was extremely like the clinical picture of coeliac disease in children. There was no evidence of rickets found in any of the kittens post mortem, but the experiments appear to indicate that kittens when given a

diet deficient in fat-soluble *A* develop a condition similar to that found in guinea-pigs and rats under the same circumstances, and that kittens are probably more sensitive than young rats to a partial deficiency of the fat-soluble accessory food factor.

In conclusion I should like to offer my sincere thanks to Dr C. J. Martin for his stimulating assistance and advice. The greater part of the laborious task of feeding the animals was carried out by Miss H. Henderson Smith, and to her as well as to Miss S. Rutherford and others who shared in the work I owe the possibility of carrying out the experiments.

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IV. THE EFFECT OF A DIET DEFICIENT IN ANIMAL FAT ON THE BONE TISSUE (RIB JUNCTIONS) OF KITTENS.

BY FRANCES MARY TOZER.

From the Department of Experimental Pathology, Lister Institute.

(Received November 15th, 1920.)

THE following remarks are based upon the examination of the rib junctions of the kittens used by Dr H. Mackay, in the foregoing experiments [Mackay, 1921].

Dr Mackay kindly supplied me with the material before giving particulars of the treatment of each animal in order that the diagnosis of the condition of the bone tissue might be unbiassed.

The results of the examination are as follows:

1. The rib junctions of the *control animals* Nos. 2, 3 and 9, were all diagnosed as "normal" with a reservation in the case of No. 3 that the bony trabeculae appeared to be slightly shortened and less normal in appearance than those of No. 2.

It will be observed on reference to Dr Mackay's paper that No. 3 was poorly nourished whereas No. 2 was well nourished. The difference in general condition is possibly sufficient to account for and explain the slight difference in histological condition.

2. The *experimental animals* (Nos. 1, 4, 5, 6 and 7). None of these animals was diagnosed without comment as "normal." No. 1 showed a very slight disorganisation of the bony trabeculae, a pronounced and possibly abnormal increase in length of the rows of cartilage cells and a slight degree of atrophy of the marrow.

Nos. 4, 5 and 7 showed considerable shortening of the bony trabeculae, slight shortening of the rows of cartilage cells and varying degrees of atrophy of the marrow—doubtful in 4 and 5 considerable in 7.

No. 6 was nearly normal except for the condition of the marrow which resembled No. 7.

The abnormalities at the costo-chondral junction in these kittens do not appear to resemble those characteristic of rickets, the only suggestion of any connection between the two conditions being the possibly abnormal length of the rows of cartilage cells in No. 1. They do however correspond closely with the changes seen on examination of the rib junctions of guinea-pigs fed on diets deficient in fat-soluble A.

That deprivation of fat-soluble *A* produces marked changes at the costochondral junction in the guinea-pig and that these changes are moreover indistinguishable from those caused by definite, but not severe, scurvy was suggested in a previous communication [Delf and Tozer, 1918]. This suggestion has since received confirmation and a fuller account of the experiments leading to this confirmation will be published shortly in the *Journal of Pathology and Bacteriology*.

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The Effect on the Guinea-Pig of Deprivation
of Vitamin A and of the Antiscorbutic
Factor, with Special Reference to the
Condition of the Costochondral Junctions
of the Ribs

BY

FRANCES M. TOZER

*From the Department of Experimental Pathology,
Lister Institute, London, S.W.*

THE EFFECT ON THE GUINEA-PIG OF DEPRIVATION OF VITAMIN A AND OF THE ANTISCORBUTIC FACTOR, WITH SPECIAL REFERENCE TO THE CONDITION OF THE COSTOCHONDRAL JUNCTIONS OF THE RIBS.*

By FRANCES M. TOZER.

*From the Department of Experimental Pathology, Lister Institute,
London, S.W.*

(PLATES XV. AND XVI.)

INTRODUCTORY.

THE experiments described below were undertaken to verify and, if possible, explain certain histological results obtained during the prolonged work on experimental scurvy which was begun by Dr Harriette Chick and her collaborators (1917-20¹⁻¹²) at the Lister Institute in 1916. In these researches numerous substances were tested for their antiscorbutic properties by means of feeding experiments on guinea-pigs, and it was considered advisable that I should make a routine examination of the condition of the bone tissue at the costochondral junction of the ribs of the animals used, in support of the clinical diagnosis. As a result of this examination it became apparent that, on the basal diet used for the early experiments, scurvy was complicated to a considerable extent by the occurrence of abnormalities due, not necessarily to deprivation of antiscorbutic, but to a deficient supply of the fat soluble A growth factor (M'Collum and Davis, 1915¹³).†

I drew attention to this complication in 1918⁽⁸⁾ and pointed out that histological changes indistinguishable from those usually considered typical of definite, but not severe, scurvy were frequently observed at the rib junctions of animals which had been fed on diets richly supplied with antiscorbutics, but

* Received February 4, 1921.

† Following the suggestion put forward by Drummond (1920¹⁴), I have adopted the term "vitamin A" instead of that of "fat soluble A," given to this principle by M'Collum and Davis.

poor in vitamin A. M. Mellanby, at about the same time, described abnormalities in tooth formation from the same cause (1918¹⁵).

The basal diet adopted by Holst and his co-workers (1912^{16, 17}), in their now classical study of scurvy in the guinea-pig, did not include a supply of vitamin A other than the small quantity presumed to be present in the oats, bran, and other grains of the basal diet or contained in certain of the antiscorbutics used, such as cabbage (1916¹⁸, 1917¹⁹, 1919²⁰, 1920²¹). There was at that time, however, no reason to suppose that any of the symptoms observed during the life of their experimental animals, or any of the changes seen at the costochondral junction might be caused otherwise than by scurvy alone. It is therefore to be inferred that in much of the work on scurvy, such as that of Ingier (1915²²), that has intervened between 1912 and the present time, the pathological picture of scurvy is confused to an unknown extent with that of another deficiency disease resulting from deprivation of vitamin A.

At the Lister Institute, as elsewhere, the necessity for supplementing the basal diet of oats and bran with some food richer in vitamin A was not at first appreciated, and a few of the earliest experiments were accordingly complicated from the point of view of pathological histology. It was the result of experiments to test the antiscorbutic properties of boiled onion which gave the first indication that some cause other than absence of antiscorbutic could produce the fragility of bone and the histological abnormalities observed. This conclusion was confirmed later by results of experiments with large doses of orange and lemon juices.

As soon as the necessity for improving the diet of oats and bran and water was appreciated, the animals were supplied with a daily ration of 60 c.c. milk (in which the antiscorbutic properties were destroyed by autoclaving for one hour at 120° C.), instead of water. As a result of this they made better growth at the beginning of experiments on scurvy, and were less liable to illness from other causes although the course of the scurvy was otherwise unaffected (1918⁵). Subsequently it was found advantageous to increase the autoclaved milk by at least 50 per cent.

Other investigators, such as M. H. Givens and B. Cohen (1918²³), B. Cohen and L. B. Mendel (1918²⁴), and M. H. Givens and H. B. M'Clugage (1919^{25, 26}), have recognised that a diet of oats and bran is deficient, not only in antiscorbutic, but in other respects also, and have attempted to supply a diet complete in every way, except for the intentional omission of one vitamin.

That it is not always easy to produce perfect growth in an experimental animal is shown by the frequent records of low weights, increased susceptibility to intercurrent disease, and by the occurrence of abnormalities in the bones and teeth. The test for the adequacy of any experimental diet consists in the production of a normal animal after a period of at least three months. This must be attained with young growing animals and breeding adults, and the weight curve, general health and activity, and the condition observed at autopsy and on histological examination should be the same as those of animals of the same species on a natural, normal diet kept under the same

laboratory conditions. Further, it should be possible to omit or reduce the accessory food factor under consideration without interfering with the diet in any other way.

The following is an account of experiments undertaken with a view to discriminating the effects on the guinea-pig of deprivation of vitamin A from those caused by lack of antiscorbutic.

For convenience these experiments are discussed under the following headings:—

- A. The experimental control guinea-pig.
- B. The effect of deprivation of antiscorbutic on the guinea-pig.
- C. " " vitamin A " "
- D. The effect of simultaneous deprivation of antiscorbutic and vitamin A on the guinea-pig.

Technique of Animal Experiments.

These experiments, with few exceptions, were started with young growing animals of between 290 and 350 grms. weight. Each animal was kept in a separate cage on a litter of peat. The animals were weighed three times a week, or more often if necessary, and the weights charted. The basal diet of oats and bran was common to all the experiments, and the sources of supply of the two accessory factors under consideration were not included in the basal diet and were, as much as possible, separate for each factor. The animals received their supply of antineuritic (the "water soluble B" growth factor of the American investigators) from the oats and bran of the basal diet.

The antiscorbutic used was orange juice. The choice of orange juice for this purpose depends on the fact that the small doses (1.5 to 10 c.c.) found sufficient to prevent scurvy are believed to contain no appreciable amount of vitamin A. This conclusion is based on the observation that there is actual loss of weight at the end of the experiments, lasting from thirty to eighty days, on animals given orange juice as antiscorbutic with no other source of fat soluble A. The vitamin A was supplied in the form of milk autoclaved for one hour at 120°C. This has been proved experimentally to contain a negligible quantity of antiscorbutic (1917¹, 1918⁵). Animals receiving antiscorbutic were given the measured dose by means of a pipette, and those having autoclaved milk were fed in the same way with part of the quantity if they did not take it readily.

Animals were killed at intervals of a few days from ten to thirty-one days, and some were kept alive for longer periods, up to one hundred days. An autopsy was made on every animal. Attention was particularly directed to observing the general condition, amount of fat present, occurrence of hæmorrhages, comparative strength of the long bones and jaw, presence or absence of fractures of the long bones and ribs, state of the teeth and condition of the internal organs. Any indications of death from other disease were noted.

Technique of Histological Examination.

The ribs of all the animals were kept for histological examination. Longitudinal sections of two or more ribs from each animal were examined, and if the ribs of an animal varied in appearance macroscopically the most normal and the most abnormal looking examples were chosen. One rib was always cut at right angles to the flat surface of the rib, the other parallel to the flat surface of the rib. The material was fixed in 10 per cent. formalin, and decalcified in 5 per cent. nitric acid in 70 per cent. alcohol: this was usually complete in one

to four days. Dehydration, embedding in paraffin, and section cutting were carried out according to the usual methods. The slides were stained with Ehrlich's hæmatoxylin followed by a mixture of rubin S (one part) and orange G (two parts), saturated solutions of both diluted after mixture according to convenience. The cartilage is stained blue, bone red, and blood corpuscles and connective tissue yellow.

RESULTS OF EXPERIMENTS.

(A) The Experimental Control Guinea-pig.

The production of a normal animal on the experimental diet described above is proof of the suitability of the diet for the animal in question, and supplies a standard of comparison for other guinea-

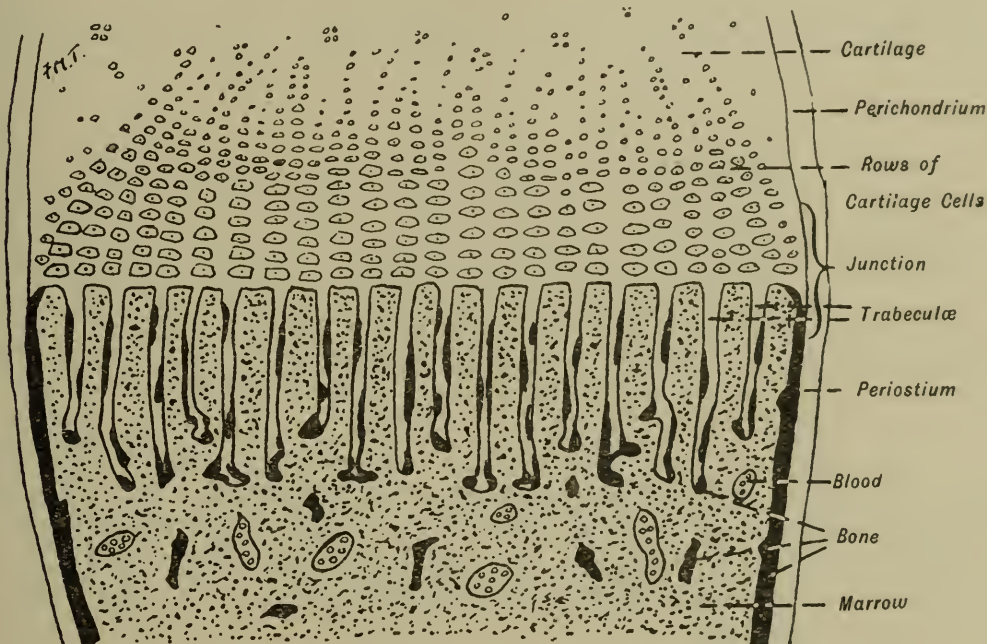


FIG. 1.—NORMAL RIB JUNCTION.

Shows the condition of the rib junction:—(i.) Of normal guinea-pigs; (ii.) of the experimental control guinea-pig; (iii.) after deprivation of antiscorbutic from 1 to 14 days; (iv.) after deprivation of vitamin A from 1 to 10 days.

pigs kept under similar conditions, but deprived either of antiscorbutic or of vitamin A or of both. The animals were under observation for ninety days or longer in order to allow time for any unsuspected trouble due to the rather abnormal diet for guinea-pigs to develop. The basal diet was given *ad libitum*, and the accessory factors in amounts experimentally proved capable of securing a normal state of health.

Condition of the Animals during Life.—No symptoms of ill-health were observed in the animals during life, and the final weight was over 550 grms. in every case. (Weight Chart I.)

Post-mortem Examination.—No abnormalities were detected.

Histological Examination of the Rib Junctions.—The costochondral junction shows the condition characteristic of that of the normal guinea-pig of this age (Fig. 1). The junction is nearly straight or

slightly concave towards the marrow cavity, the rows of cartilage cells and the trabeculae are typically regular in arrangement; there is no deformity at the junction and no bending or fracture of the bone. A closely packed mass of marrow cells reaches up to and fills the intervals between the trabeculae, and there are no patches of connective tissue in the marrow cavity.

(B) The Effect of Deprivation of Antiscorbutic.

A normal animal having thus been produced on a diet in which the two accessory factors under consideration were supplied separately, the one in orange juice the other in autoclaved milk, it is now proposed to describe the results which follow the absence of one or other of them. It was always arranged that the one supplied was consumed in amounts well above the minimum. In drawing conclusions therefrom, the possibility of some beneficial interaction of these accessory factors must not be lost sight of, but since no conclusive experiments have, as yet, been made to determine how large a part, if any, this possible interaction plays in the production of the normal animal the question has of necessity been left out of consideration in the following experiments. In this section, therefore, symptoms observed and histological changes seen in the rib junctions after omission of the orange juice are considered to be caused solely by absence of antiscorbutic.

Condition of the Animals during Life.—The effects of deprivation of this accessory factor are not apparent during the first fourteen days of experiment, the animals appear healthy and active, and the only symptom is an occasional indication of soreness of the knee or shoulder joints. After this time, however, the disease can be recognised by characteristic symptoms which increase in severity with the length of time of the deprivation. Soreness of knee or shoulder joints is noticeable after fifteen days and a consequently decreased activity. By the twenty-third day the guinea-pig occasionally adopts the "scurvy position" in which it "rests on its side while the painful member is held twitching in the air" (1919²⁷). Between the twenty-third and thirtieth day the animal becomes seriously ill, the teeth are loose, the coat staring. The "scurvy position" is almost constant, and the "scurvy faceache position" is observed; in the latter the animal "lies down with the side of its face upon the floor of its cage indicating that its gums and jaws have become painful" (1919²⁷). The appetite fails and it becomes necessary to hand-feed it. If the disease is allowed to continue death takes place about the thirtieth to the thirty-fifth day.

The weight rises during the first part of the experiment, and the maximum is reached between fourteen to nineteen days. The drop, which occurs almost immediately after the maximum, is sudden and is so constant that it may be regarded as characteristic. (Weight Chart I.).

Post-mortem Examination.—Animals killed at ten days and at fourteen days are found to be normal, except that very slight ridges can sometimes be observed at the costochondral junction of the ribs and the limb bones can be broken on application of considerable pressure. By seventeen to eighteen days definite symptoms are seen in some cases, the bones being distinctly fragile and hæmorrhages present. The rib junctions vary considerably, some being very swollen,

others being normal in appearance. Between this time and death at about thirty days the symptoms rapidly increase in severity, the bones at twenty-eight days are very thin and fragile, the limb bones, if not fractured during life, do so upon slight pressure and separate easily at the epiphyses. The teeth are very loose and brittle, severe hæmorrhages—subcutaneous, intramuscular, periosteal and in any of the internal organs—may be present. The majority of the rib junctions are swollen and deformed, and the general condition is very poor.

Histological Examination of the Rib Junctions.

No abnormalities are detected on examination of the costochondral junction of the ribs before seventeen days. At or about this time, however, the ribs of an animal suffering from scurvy are found to be in one or other of the two conditions described below.

Ribs which are macroscopically flat and normal in appearance (see above) are in a condition in which the microscopic changes are so

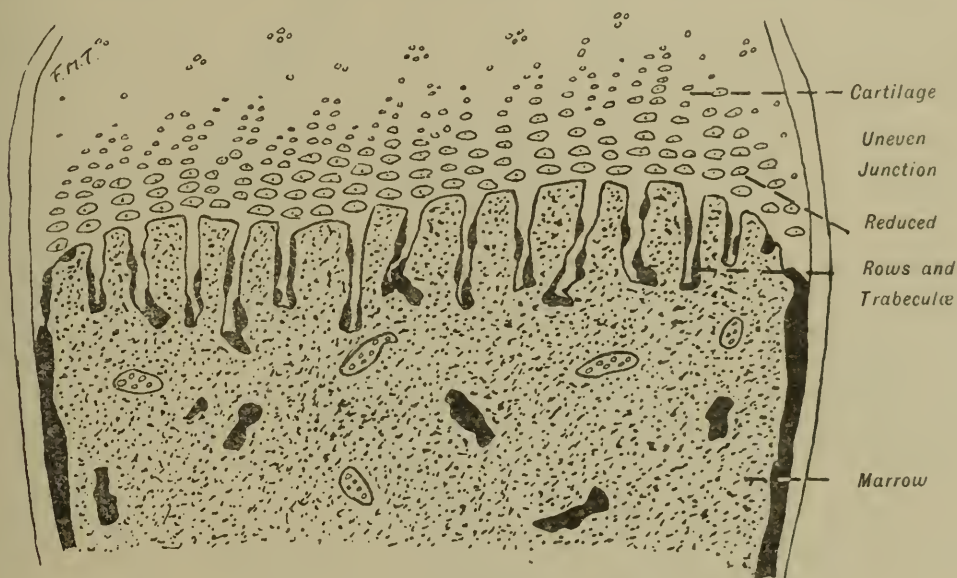


FIG. 2.—THE "INCIPIENT" STAGE.

Shows the condition of the rib junction:—(i.) After deprivation of anti-scorbutic between 14 to 21 days (in some ribs only); (ii.) after deprivation of vitamin A at 14 days.

slight that they are best described as "incipient." The most noticeable departures from the normal are a variable amount of irregularity of the junction as a whole, slight disarrangement, and usually shortening of the rows of cartilage cells and of the trabeculae; an increased amount of blood in the marrow cavity, and a reduction in thickness of the bone, especially near the junction (Fig. 2).

The swollen ribs of an animal killed at the same date (see above) are found to be in a condition in which the microscopic changes are more pronounced. The rib junction as a whole is disorganised, the rows of cartilage cells have lost their alignment and are scattered, and the trabeculae are shortened. The bone, which has become thin, is fractured on one or both sides of the junction, and, as a rule, quite

Another condition, described elsewhere (1918⁸) as "definite," is met with in cases of subacute scurvy when, owing to the administration of a certain amount of antiscorbutic the more serious symptoms have been prevented. This condition is represented in Fig. 4, but for convenience and to prevent repetition the description is reserved until later.

(C) The Effect of Deprivation of Vitamin A.

This experiment was conducted on the same lines as the preceding one, but the animals were supplied with antiscorbutic and deprived of vitamin A.

Condition of Animals during Life.—There are no symptoms of illness under twenty-one days. The animals are healthy and active though thin, but they do not grow at all. There is no definite soreness, as is found in scurvy at this time. At later periods, except for extreme emaciation and an increased liability to intercurrent disease, there are no symptoms which can with certainty be ascribed to deprivation of this accessory factor. After thirty days the liability to death from other causes increases and the general condition becomes very poor. The animals usually die under sixty days, and observations upon the condition after sixty days were, therefore, limited to one animal. There is no permanent increase of weight; any gain which occurs within the first few days is lost soon after and the final weight is always below the initial (Weight Chart I.).

Post-mortem Examination.—Animals killed at ten and fourteen days are normal except that the bones are slightly fragile and minute ridges can be detected at the rib junctions. As might be expected from the weight, they are thin and undersized, but not emaciated. The fragility of the bones increases with length of time and the teeth become brittle, but not loose, and very much worn down. No fractures are observed till a much later period, over fifty days, by which time the bones have become thin and brittle (compare scurvy). No hæmorrhages comparable with those seen in scurvy occur at any period.

Deprivation of vitamin A results, therefore, in the production of a thin, miserable creature, with fragile bones and brittle teeth, but with no other obvious distinctive symptoms either during life or at autopsy.

Histological Examination of the Rib Junctions.

Slight changes are noticeable in the costochondral junction at fourteen days. The trabeculæ and rows of cartilage cells are a little shorter and less regular in arrangement than the normal. The changes are indistinguishable from those described as "incipient" in the macroscopically normal-looking ribs of scurvy animals at a slightly later period (see p. 311). The same illustration (Fig. 2) is, therefore, used to represent both cases.

By twenty-one days the condition at the rib junction is noticeably abnormal; it does not, however, resemble that described above as "acute" or "severe" in scurvy at about the same time. The chief abnormality is the considerable reduction in length both of the trabeculæ and the rows of cartilage cells; the marrow shows slight signs of atrophy, but there is no hæmorrhage. The condition has been described as the "definite" stage (1918⁸). It so closely

resembles that observed in partially protected and chronic scurvy cases mentioned above that the same illustration represents equally well both conditions (Fig. 4).

These changes are progressive, and by thirty to forty days the rows

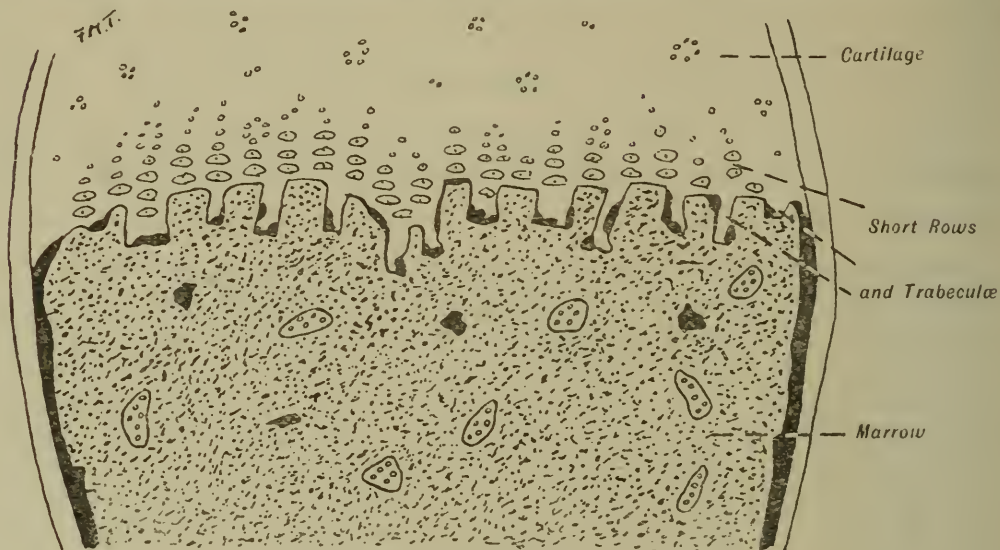


FIG. 4.—THE "DEFINITE" STAGE.

Shows condition of the rib junction:—(i.) After *partial* deficiency of anti-scorbutic at about 40 days "mild scurvy"; (ii.) after deprivation of vitamin A at 21 to 30 days.

of cartilage cells and trabeculae have almost disappeared. Progressive atrophy of the marrow also occurs, and in extreme cases little but the

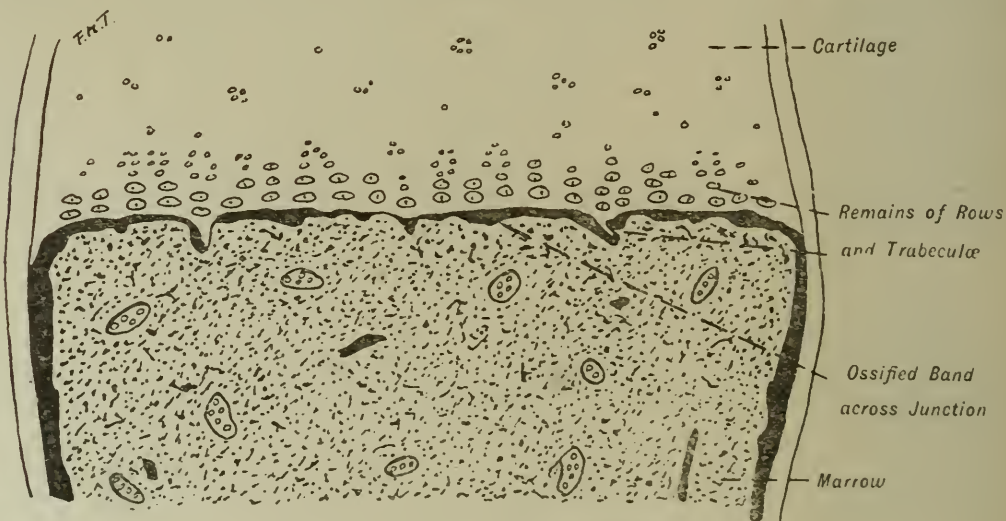


FIG. 5.—THE "DEFINITE" STAGE.

Shows condition of the rib junction:—(i.) After considerable deficiency of antiscorbutic for a long time, "chronic scurvy"; (ii.) after deprivation of vitamin A for a long time (over 50 days).

delicate reticulum of the connective tissue ground-work and the blood-vessels remains. The cartilage often ends in a thin, ossified band, which is apparently laid down in an attempt to buttress up a weak joint (Fig. 5).

After fifty days, owing to the increased fragility of the bones, fractures of some of the ribs occur and the sections show proliferation of cartilage cells and development of connective tissue in the marrow cavity (Plate XV., Fig. 1).

Here again some resemblance can be traced to the effect of scurvy, but it should be noticed that while this condition is common in acute scurvy between eighteen and thirty-five days, it is rare as a result of deprivation of vitamin A. It would seem indeed that in both cases the pronounced abnormalities, the deformity of the junction, and the mass of connective tissue are complications produced by fracture of the fragile bone rather than symptoms of either disease.

The development of connective tissue in the marrow cavity of the rib junctions of animals deprived of vitamin A as well as in the same position in cases of severe scurvy proves that its presence is not in itself a scurvy symptom, although the frequency with which it occurs in the latter disease would account for the prevalence of this idea. In scurvy, this connective tissue has, so far, only been observed in rib junctions where a fracture is obvious or is strongly suspected, that is, in all cases of scurvy severe enough to bring about fracture at any time from eighteen days until death. It is not seen in chronic scurvy where there is no evidence of existing or recent fracture. Since neither fracture nor connective tissue have been seen before eighteen days it is inferred that development is rapid and it seems probable from the few observations made upon this point that its disappearance is also rapid in cases of cure; evidence on this subject is, however, at present inconclusive. The fibrous tissue is presumably developed in an attempt to repair the fracture.

(D) The Effect of Simultaneous Deprivation of Antiscorbutic and Vitamin A.

The animals used in this experiment were fed on the basal diet of oats and bran and water alone. The results are supported by additional data obtained in previous testing of various antiscorbutics in which, as mentioned above (p. 306), vitamin A was given in amounts now known to be below the minimum required for protection.

Condition of the Animals during Life.—No symptoms are observed under fourteen days except that the animals do not grow and are very thin. The joints are tender and appear swollen after the fourteenth day, but the scurvy position is not observed any earlier than in scurvy alone. The further course of the disease is similar to that of scurvy, but the fatal termination occurs a few days earlier.

The weight curve shows the flattening characteristic of deprivation of vitamin A followed by the sudden steep drop peculiar to the scurvy curve (Chart I.).

Post-mortem Examination.—Animals killed at fourteen days show no abnormalities except poor general condition and slight fragility of the bones. Haemorrhages, fractures, brittle and loose teeth are found at about the same time as they occur in scurvy alone. The general condition when death occurs at about twenty-five days is one of extreme emaciation.

TABLE I.

	A. Experimental Control.	Time in Days.	B. Deprivation of Antiscorbutic.	C. Deprivation of Vitamin A.	D. Simultaneous Deprivation of Antiscorbutic and Vitamin A.
DIET . .	Oats, bran, <i>ad lib.</i> Orange juice 10 c.c. Auto- claved milk, <i>ad lib.</i> (Average for seven animals 70 c.c. taken daily.)	...	Oats, bran, <i>ad lib.</i> No orange juice. Autoclaved milk, <i>ad lib.</i> (Average for ten animals 75 c.c. taken daily.)	Oats, bran, <i>ad lib.</i> Orange juice 10 c.c. No autoclaved milk.	Oats, bran, <i>ad lib.</i> No orange juice. No auto- claved milk. Water, <i>ad lib.</i>
SYMPTOMS DURING LIFE	Health good . .	1 to 14	Healthy and active . .	Healthy and active . .	Healthy and active.
	Active . . .	18th and after	First signs of pain and swell- ing of joints. Activity decreased.	No definite symptoms. Ac- tive.	Sore and swollen joints.
	...	23rd and after	Scurvy position . . .	No scurvy position. Active.	Scurvy position.
	...	24th and after	Ill, crippled, coat staring . .	No symptoms; thin and active.	Ill, crippled, moribund. Death.
WEIGHT.	...	28 to 35	Death . . .	Increased liability to infection.	...
	...	35 to 100	...	Death
	...	1 to 14	Rising . . .	Slight rise, stationary or falling.	Stationary or falling.
	Rising from 300 grms. to over 600 grms. in 90 days.	14 to 18	Maximum . . .	Falling . . .	Rapid fall begins.
	...	18 to 30	Rapid fall; final is below initial weight.	Stationary or falling . .	Rapid fall.
	...	30 to 100	...	Continued slight fall. Final is below initial weight.	...

Histological Examination of the Rib Junctions.

Slight abnormalities resembling those seen after deprivation of vitamin A alone can sometimes be detected at fourteen days. At later dates, eighteen to twenty-five days, the condition appears to be the same as that caused by scurvy alone—that is, normal looking, unfractured ribs show slight changes only, while fractured ribs show the characteristic scurvy hæmorrhage, disorganisation of the junction and masses of connective tissue.

One animal, which was adult at the beginning of the experiment, died at twenty-seven days. There were few symptoms during life, and the severe hæmorrhage reported at post-mortem was of internal organs only. Unfortunately only two ribs were examined after the death of the animal in December 1916 and no more are available.

Both these ribs are in the same condition. The trabeculæ and rows of cartilage cells have nearly disappeared, the marrow is normal, there is no sign of either a fracture or of the development of connective tissue in the marrow cavity. This absence of the more serious symptoms is due, in all probability, to the greater age and strength of the animal at the beginning of the experiment.

DISCUSSION OF RESULTS.

The results of the foregoing experiments are epitomised in tabular form in Table I. They show clear evidence that unless the scurvy animal is protected from the effects caused by insufficient vitamin A the results will be complicated and the diagnosis may be incorrect. The data obtained from these experiments make it possible, however, to point to certain symptoms as being those of scurvy and not the effect of deprivation of vitamin A. The clinical picture in guinea-pigs of deprivation of antiscorbutic alone is graver than that of vitamin A. Severe illness and death are inevitable in the former between twenty and thirty days. Death as the direct result of the latter alone occurs only after a much longer experimental period, and as a rule is due to intercurrent disease. No guinea-pigs on a diet completely deficient in vitamin A have been kept alive for longer than one hundred days.

Loose teeth, tender and swollen joints and the scurvy position are signs of true scurvy; the occurrence of hæmorrhage, especially in the limbs, is a most constant characteristic. Fractures also may be regarded as a symptom of scurvy, they occur at an earlier stage in this complaint, *i.e.*, eighteen to thirty days, whereas they have been observed only after prolonged total or considerable deprivation of vitamin A.

Simultaneous deprivation of both these accessory factors does not seem to accelerate the onset of the scurvy symptoms, but after these appear they mask the less severe symptoms caused by absence of

vitamin A, so that the general character of the disease appears to be that of scurvy. Death, however, in these cases usually takes place earlier, at about twenty-four days, than when caused by scurvy alone. The weight curve shows the typical scurvy drop as well as the flattening due to deprivation of vitamin A (Weight Chart I.). The diagnosis of scurvy or of disease caused by deprivation of vitamin A from the microscopic appearance at the costochondral junction is affected in the same way as that based on observation of the condition at autopsy, that is by the fact that severe scurvy symptoms obscure those caused by deprivation of vitamin A.

It is possible to diagnose a case of acute scurvy from the observation

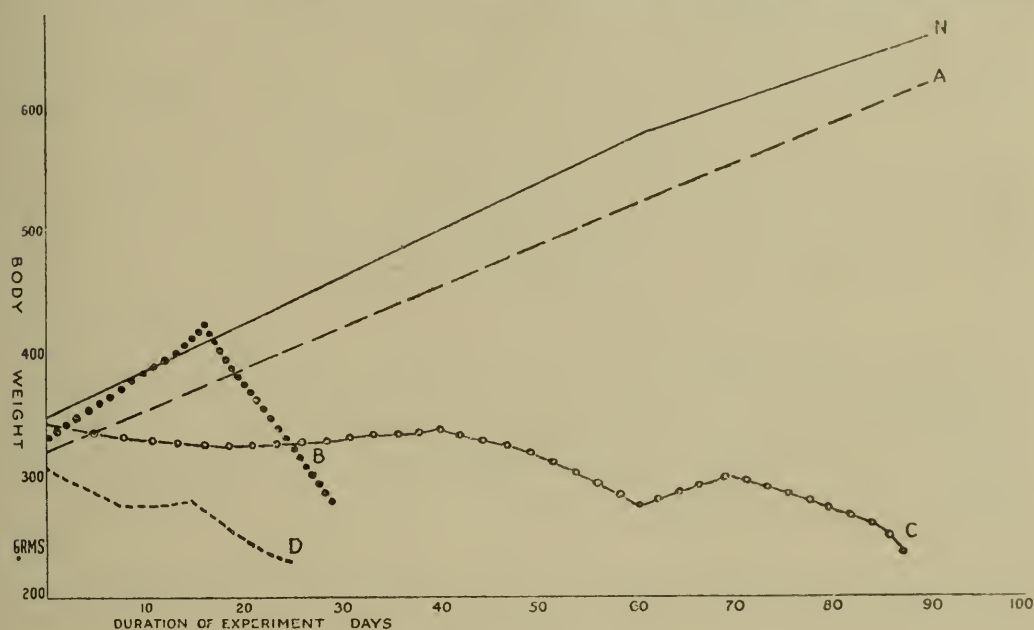


CHART I.

- N = Mean weight chart of four animals on normal diet of oats, bran, and 30 grms. of fresh cabbage.
 A = Mean weight chart of five animals on experimental normal diet of oats, bran, autoclaved milk and orange juice.
 B = Mean weight chart of eight animals on oats, bran, and autoclaved milk.
 C = Mean weight chart of two animals till fifty days (one after fifty days) on oats, bran, and orange juice.
 D = Mean weight chart of five animals on oats, bran, and water.

of hæmorrhage into the marrow cavity, fracture of the bone, and the presence of connective-tissue in the region of the junction, but, without reference to the data regarding the supply of vitamin A, it is impossible to say whether such acute cases of scurvy are, or are not, complicated by deficiency of vitamin A.

Further, it is not possible, from the histological appearance alone, to distinguish with certainty mild or chronic scurvy (*i.e.*, cases which have been partially protected from the disease by the administration of small amounts of antiscorbutic) from cases of deprivation of vitamin A; nor can one tell, from observation of the bone-tissue alone, whether an animal has suffered slight simultaneous deficiency of both accessory factors. The abnormalities which occur first in

both diseases appear to be of the same character and are indistinguishable from each other for a short time after they first appear. These slight abnormalities are manifest at an earlier date after the commencement of deprivation of vitamin A than after deprivation of antiscorbutic. In a parallel manner growth ceases almost at once after deprivation of vitamin A, whereas it continues for twelve to nineteen days after deprivation of antiscorbutic. If deprivation of vitamin A continues, the character of the abnormalities at the junction of bone and cartilage does not change until a late period, but the trabeculae appear shorter and the marrow more atrophied.

The character of the changes at the junction when deprivation of antiscorbutic continues is on the contrary altered or obscured by the

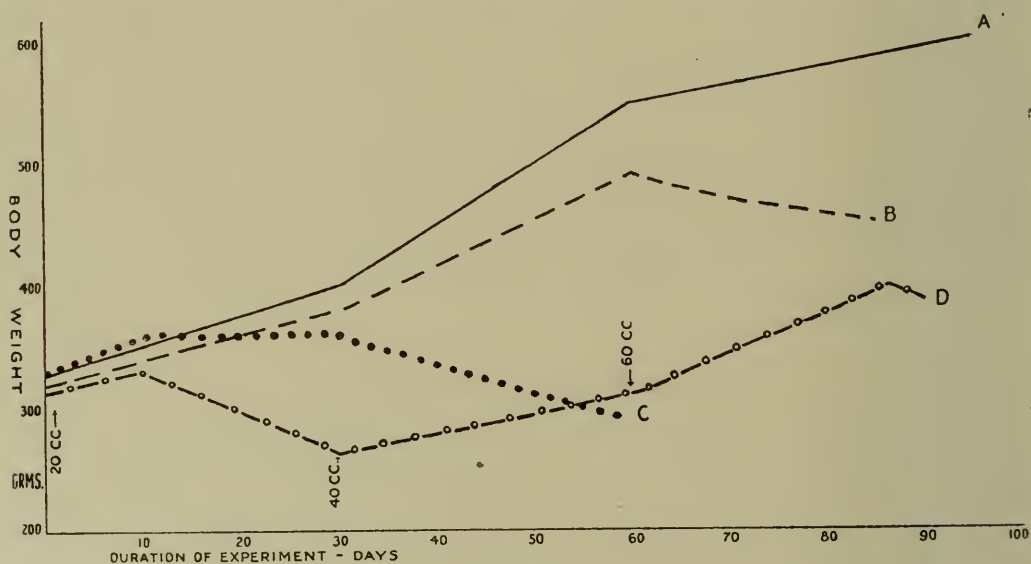


CHART II.

- A = Weight chart of case of chronic scurvy showing serious symptoms at autopsy. Consumption of autoclaved milk good. 87 c.c. average.
 B = Mean weight chart of two cases of severe chronic scurvy. Consumption of autoclaved milk good. 79 c.c. average.
 C = Weight chart of case of severe chronic scurvy. Consumption of autoclaved milk poor. 43 c.c. average.
 D = Weight chart of a case of partial deficiency of Vitamin A showing improved growth on increase of autoclaved milk. (Antiscorbutic adequate 10 c.c. orange juice.)

early occurrence of complications in the shape of hæmorrhage and fracture. These complications result in the disorganised junction which has been recognised by all observers as typical of severe scurvy. The condition of the marrow is, however, of some assistance in determining doubtful cases. Numerous observations lead me to believe that deprivation of vitamin A is followed by atrophy of the marrow. Slight wasting has been observed twenty-one days after deprivation of vitamin A, whereas the same condition has not been seen in scurvy when the consumption of autoclaved milk has been adequate. In cases where atrophy of the marrow has been observed in chronic scurvy the adequate supply of vitamin A is usually open to suspicion. Careful scrutiny of the weight curve, of the amounts of vitamin A

and of antiscorbutic consumed is therefore necessary before a correct diagnosis can be arrived at.

As already mentioned, the pioneer work of Holst and Frolich (1912¹⁶) was undertaken at a time when there was little or no available information concerning the importance of vitamin A for normal growth, consequently their important investigation was conducted on lines which, as regards many of their experiments, did not secure an adequate supply of this vitamin for their experimental animals. The study of their data concerning diets and weights shows that, as in the earliest of the experiments at the Lister Institute, the animals were affected by this deprivation. In consequence of this the histological condition they describe is best compared with that referred to above in section C (deprivation of both antiscorbutic and vitamin A).

The diagnosis of scurvy made by these investigators depends principally on looseness of the teeth and the occurrence of hæmorrhage, and is certainly correct, though possibly incomplete, when these symptoms have been observed. That a diagnosis of mild scurvy would be correct when these symptoms have not been observed, is now, I think, open to question.

Sir Thomas Barlow (1894²⁸), in his Bradshaw lecture of 1894, which might have been written yesterday, also lays stress on the occurrence of fractures and hæmorrhage as the essential distinguishing feature of scurvy in human infants. He points out that these symptoms are absent in rickets and that in consequence the pathology becomes complicated when scurvy supervenes "on a basis of rickets."

There are a number of observations, some old, some recent, pointing to deprivation of vitamin A as in some way concerned in the occurrence of rickets. Bland Sutton (1884^{29, 30, 31}) observed the development of rickets (or osteomalacia, according to age), in gerbills and in monkeys kept in captivity. He also made experiments on lion cubs at the Zoological Gardens and was able to prove that rickets, in these animals, could be prevented by an adequate diet.

According to Mellanby (1919³²), rickets in dogs is a disease resulting from a deficiency of an "anti-rachitic factor," which is present to a considerable extent in animal fats. This last fact has suggested its possible identity with vitamin A (1919²⁷). Hess (1920³³), on the contrary, while believing that rickets is "primarily a dietetic disorder" and that cod-liver oil, a fat rich in vitamin A, is a valuable agent in the prevention and cure of this disease, does not consider that deprivation of this accessory factor is the primary cause of rickets in infants.

In a previous paper Hess and Unger (1918³⁴) describe an appearance which they observed at the rib junction of the guinea-pig in the following words: ". . . We have encountered macroscopic lesions at autopsy, which we considered characteristic of true guinea-pig scurvy (hæmorrhage into the costochondral junction of the ribs and swelling of the joints), but which later microscopic examination proved to resemble rickets. . . ." The possibility that deficiency of vitamin A in the diets of these scurvy guinea-pigs may play some part in the production of this peculiar condition is not suggested by the authors, but it should not, I think, be lost sight of.

Mackay (1921³⁵) attempted to produce rickets in kittens on a diet deficient in animal fat, but was unable to do so. On examination of the rib junction of Dr Mackay's animals I found³⁶, however, changes closely resembling those which I have described above in the guinea-pig under similar conditions.

None of my guinea-pigs which were deprived of vitamin A (Section C) exhibited the general pathological picture of rickets as

seen in children in which softening of bones and considerable disorderly growth at the epiphysis with the development of osteoid tissue is characteristic. The animals failed to develop, and their general condition was very thin and poor. Osteogenesis seemed to be in abeyance. Both at the epiphyseal line and at the costochondral junction all indications of activity had diminished or disappeared, and the bones had become thin and brittle.

Some of the sections from animals which have survived deprivation of vitamin A for over sixty days, and also from cases of chronic scurvy complicated by deficiency of vitamin A, do, however, show a disorganised activity at the costochondral junction (Plate XV., Figs. 1-3, Plate XVI., Figs. 4, 5).

Instead of the orderly progression—cartilage cells proliferating so as to form rows at right angles to the junction, blood-vessels carrying osteoblasts making their way into the spaces occupied by these cells with the consequent formation of trabeculæ which rapidly calcify—the whole process is disorderly. The cartilage cells cease to increase in their normal manner, the rows disappear, and with their disappearance the guiding influence for the entering blood-vessels, which the cartilage cells normally possess in virtue of their arrangement in rows, is lost, and the blood-vessels enter irregularly.

Cartilage cells are thus cut off and remain as islands in the marrow cavity. These islands are sometimes seen at some distance from the junction, and their position probably indicates the position of the junction itself some time previously (Plate XVI., Fig. 6).

These are, however, the changes which were epitomised by Morpurgo (1907³⁷) when presenting the important results of his seven years' work on an infective form of rickets in rats at a meeting of the German Pathological Society in 1907, as follows:—"The most important appearance to which attention should be paid is the defective development in the preparatory calcification of the cartilage. The "arrows" (trabeculæ) of calcified cartilaginous substance disappear, and the blood-vessels, ascending from the marrow at the boundary of the cartilage, deviate laterally from the calcified towards the uncalcified parts. The cartilage junction appears in longitudinal sections "wavy" not "thorny" as under normal conditions, and the sub-cartilaginous trabeculæ are scantily and poorly developed." There is nothing in this description which could not also be applied to the exceptional cases first referred to. Investigation into the circumstances attendant on their occurrence has, as yet, however, revealed no factor, dietetic or other, common to them all. The condition occurs, not only when both factors are deficient in the diet, but also, less frequently, when vitamin A alone is lacking.

In conclusion I should like to express my sincere thanks to Professor C. J. Martin for his kind advice and assistance; to

Dr H. Chick and all her collaborators in the scurvy investigation for putting all the histological material at my disposal for use in this paper, and to Miss M. Rhodes, Miss S. Rutherford, and Miss H. Henderson Smith for their invaluable help in the tedious work of feeding the animals. Some of the preliminary observations were made at University College Hospital Medical School with the assistance of the Graham Fund of the University. The expenses of the investigation have been partly defrayed by a grant from the Medical Research Council. Text-figures 1-5 are reproduced by permission of the Editors of the *Biochemical Journal*.

SUMMARY.

1. The results are recorded of experiments on the guinea-pig which were devised to investigate and distinguish between the effects produced by deprivation of antiscorbutic, of vitamin A, or of both these accessory factors together, with special reference to the histological condition of the bone tissue.

2. The suggestion is confirmed—that deprivation of vitamin A has a pronounced effect on the bone tissue, indistinguishable, under certain conditions, from the effects of scurvy.

3. An attempt is made to define true scurvy symptoms as shown during life, at autopsy and from histological examination, and to distinguish them from symptoms caused by absence of vitamin A.

4. Histological appearances at the costochondral junction bearing some resemblance to rickets are described and illustrated. These peculiar abnormalities have been observed in animals suffering from prolonged deprivation of vitamin A, and from mild and from acute scurvy complicated by deficiency of vitamin A.

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DESCRIPTION OF PLATES.

PLATE XV.

FIG. 1.—Condition of costochondral junction of guinea-pig No. 631 after prolonged deprivation of vitamin A. This animal was fed on a diet of oats and bran *ad lib.*, 10 c.c. fresh orange juice daily, and 60 c.c. of autoclaved milk daily from the twenty-eighth to the forty-sixth day of experiment only. Water was given instead of milk for the remainder of the experiment. The animal died after eighty-nine days. Attention is directed to the extreme atrophy

of the marrow (white in photograph), the fracture, connective tissue (greyish), and proliferation of cartilage. (See p. 315.)

- a. Blood (dark).
- b. Reticulum of fibrous atrophied marrow (white).
- c. Connective tissue 'greyish'.
- d. Abnormally proliferating cartilage.
- e. Fracture.

FIG. 2.—Condition of costochondral junction of guinea-pig No. 67, after deprivation of vitamin A. The animal was fed on a diet of oats and bran *ad lib.*, 15 grms. of boiled onion and water. It died after fifty-five days. The marrow is atrophied, but not to the extreme degree of the animal above. (See p. 322.)

- a. Marrow (atrophied).
- b. Abnormally proliferating cartilage.
- c. Fracture.

FIG. 3.—Condition of costochondral junction of guinea-pig No. 447 after simultaneous deficiency (not total deprivation) of antiscorbutic and vitamin A for fifty-six days. The animal received oats and bran *ad lib.*, 1.5 grms. germinated lentils, and an average of 28 c.c. daily of autoclaved milk. Attention is directed to the islands of cartilage. (See p. 322.)

- a. Islands of cartilage.

PLATE XVI.

FIG. 4.—Shows the condition of the costochondral junction of a guinea-pig after some deficiency of vitamin A, 40 c.c. autoclaved milk daily (about two-thirds the minimum). Antiscorbutic 5 c.c. fresh lemon juice daily (about three times the minimum). (See p. 322.)

- a. Bone.
- b. Marrow, nearly normal (dark).
- c. Trabeculae absent, zone of ossification in disorder.
- d. Island of cartilage.

FIG. 5.—Shows the condition of the costochondral junction of a guinea-pig after considerable deficiency of antiscorbutic, cabbage heated at 90° C. for two hours, 5 grms. daily (see Delf⁸) and some deficiency of vitamin A (about two-thirds the minimum). The animal died on the fifty-ninth day of experiment. (See p. 322.)

- a. Marrow, normal (dark).
- b. Bone.
- c. Abnormal bone tissue.
- d. Trabeculae and rows of cartilage cells absent. Zone of ossification in disorder.

FIG. 6.—Shows the condition of the costochondral junction of a guinea-pig after considerable and prolonged (seventy-seven days) deficiency of vitamin A. For the first sixteen days of experiment no autoclaved milk was given; after this date 31 c.c. per day was consumed (about half the minimum). Antiscorbutic—10 per cent. fresh lemon juice daily (much above the minimum).

- a. Marrow, atrophied (light).
- b. Island of cartilage indicating previous position of junction.
- c. Disappearance of normal rows and trabeculae.

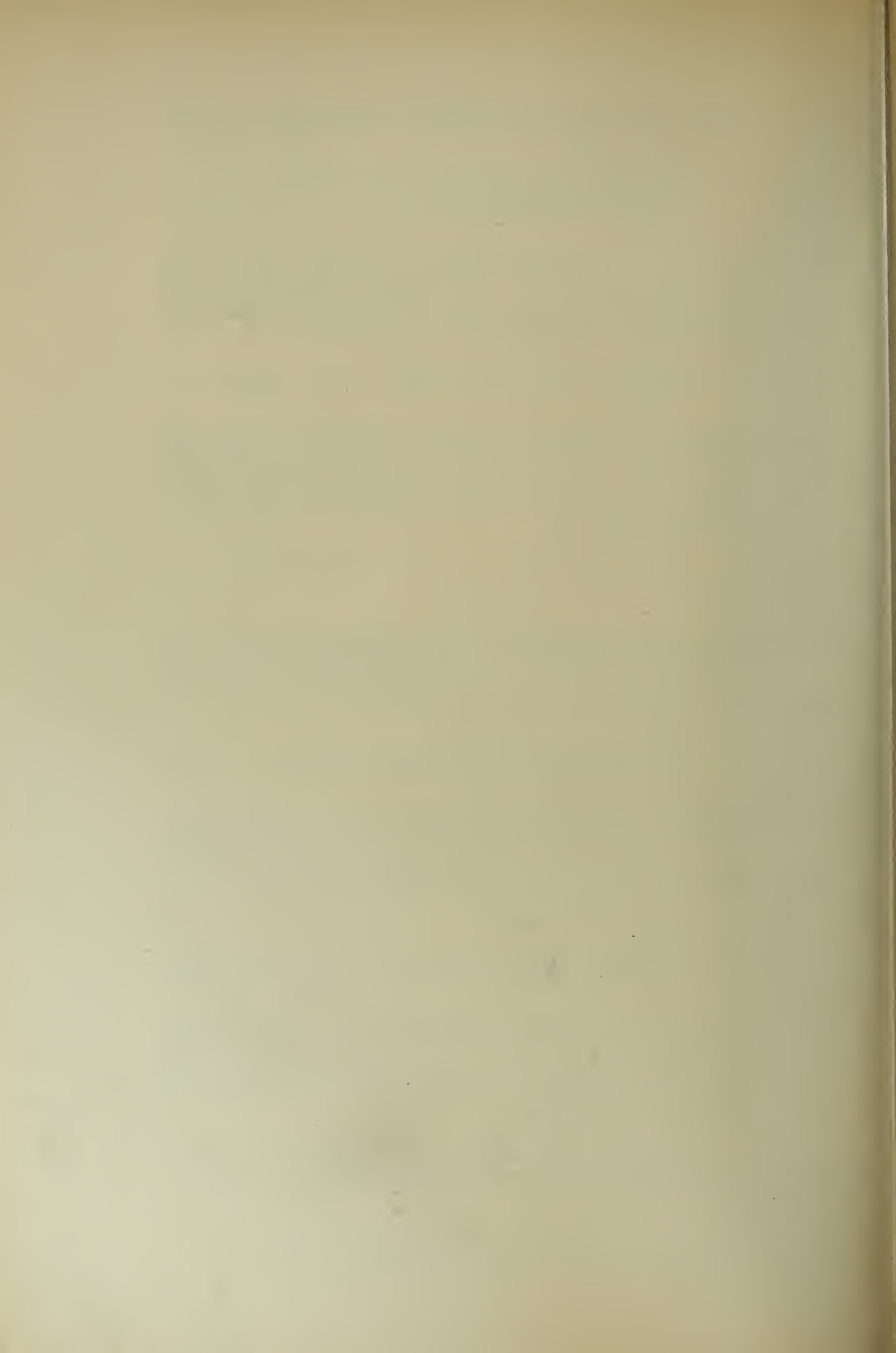


FIG. 1.

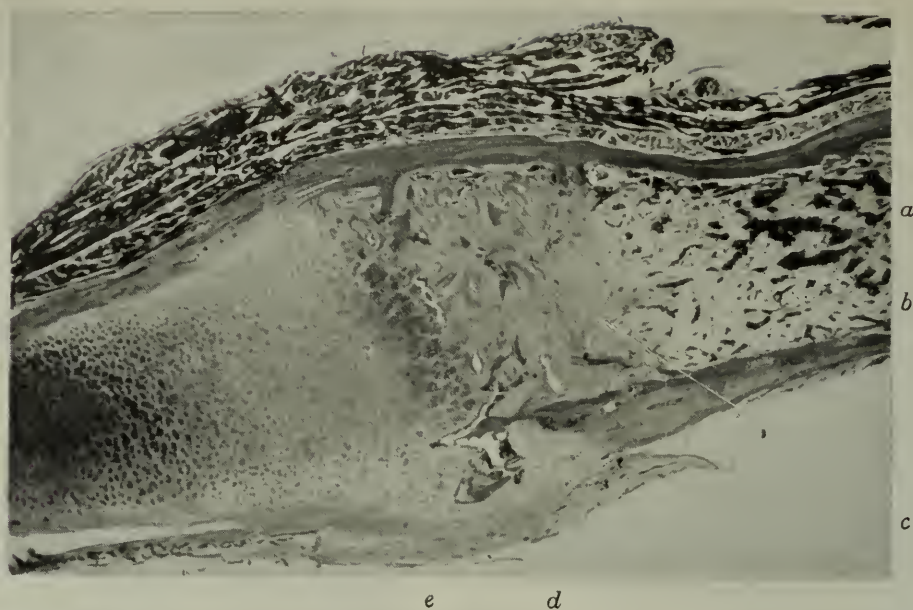


FIG. 2.

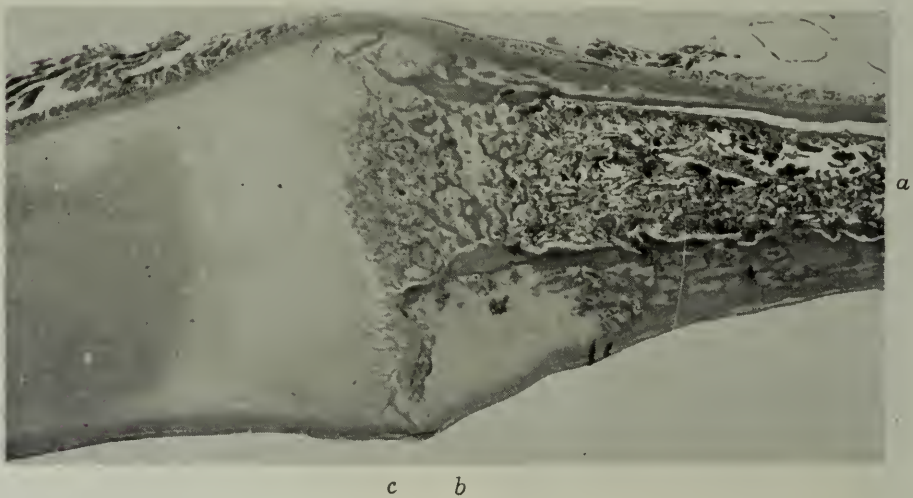


FIG. 3.

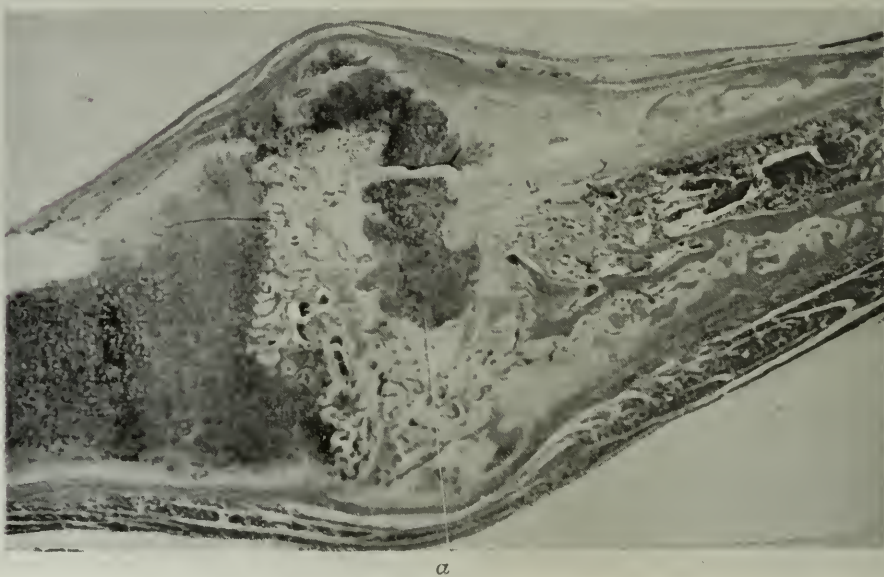


FIG. 4.



FIG. 5.

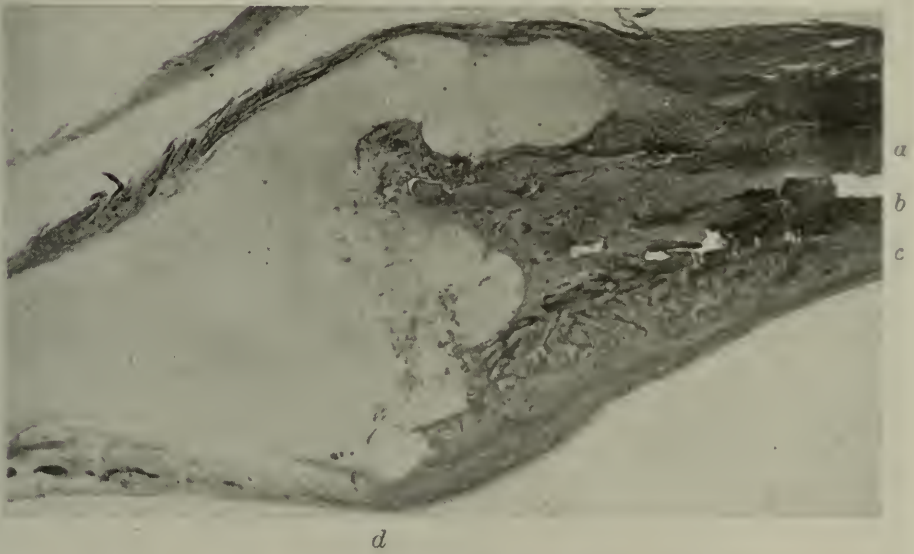
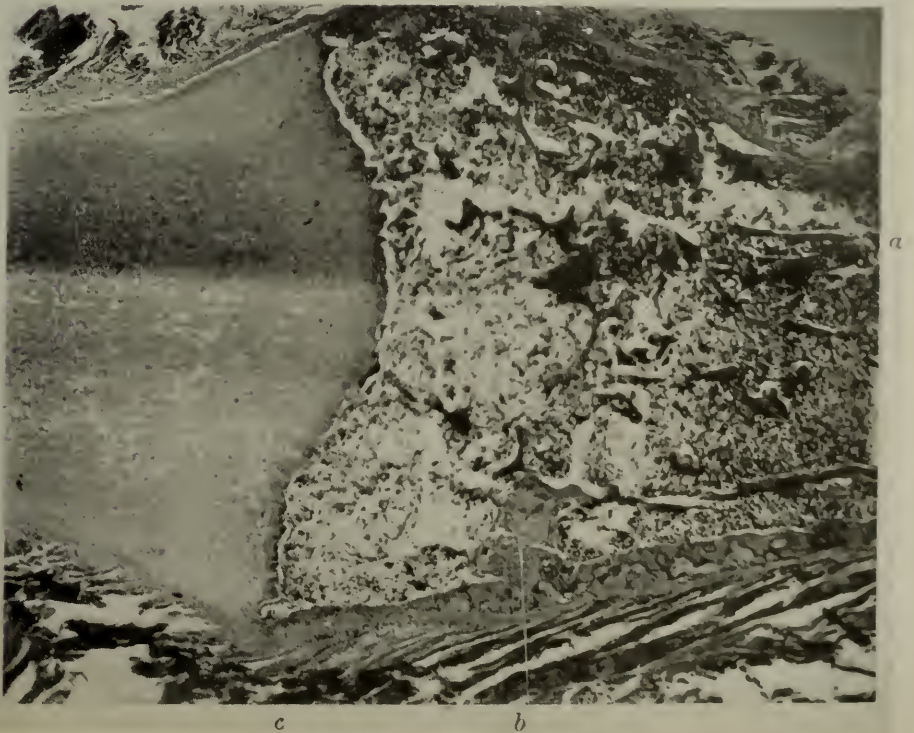


FIG. 6.



XLI. THE EXTRACTION OF THE FAT-SOLUBLE FACTOR OF CABBAGE AND CARROT BY SOLVENTS.

BY SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute.

(Received April 29th, 1920.)

THE study of the fat-soluble accessory factor in fats has so far not contributed anything very definite to our knowledge of the chemistry of this principle. Observations so far made on active fats favour the view that the activity is not due to the fat itself but to some substance or substances which are in association with it. The fat-soluble factor is however not easily separated from it by differential solvents. All other conceivable methods of fractionation are mostly of a drastic nature not suitable for the manipulation of a physiologically active principle of doubtful stability.

It is now well established that some vegetables form a rich source for a substance physiologically resembling the fat-soluble accessory factor in fats but as in the case of the fats little is known of its chemical character. We are even yet unaware whether it is present in the vegetable cell in solution or in suspension. According to McCollum, Simmonds and Pitz [1916, 1] the fat-soluble factor is not extracted from plants with the fats by ether, chloroform, benzene or acetone. Hot alcohol however according to these authors [1916, 2] removes the factor from maize kernel.

This communication describes some experiments in which active fractions were obtained from fresh vegetables by extraction with absolute alcohol and eventually with ether.

Originally it was intended to study the fat-soluble factor in cabbage but owing to some technical difficulties the investigation was extended to carrots. It has been found by various workers that cabbage is rich in the fat-soluble factor. Preliminary experiments carried out in connection with this research have shown that small quantities of this vegetable in fresh condition were sufficient to promote growth in rats fed on a diet lacking the factor. The aim was to treat the minced cabbage at first with a solvent which would fulfil the dual function of disorganising the plant cells and of extracting the accessory factor. Absolute alcohol was chosen and found to be suitable for the purpose. However the extract thus obtained was not relished by the rats who mostly either refused the food containing it or consumed insufficient

of it. It was therefore found necessary to apply this method of extracting to another vegetable, namely carrots, the extract of which was likely to prove more palatable to the animals. This vegetable although not very rich in the fat-soluble accessory factor was shown by Denton and Kohlman [1918] and Steenbock and Gross [1919] to contain appreciable quantities of it. As anticipated it was found possible to extract by means of alcohol from the carrots an active fraction which the animals took well. This extract afforded the opportunity of further fractionation, and it was found that ether extracted the active principle from it leaving the best part of the extraneous matter behind. As the carrot also contains the antineuritic and antiscorbutic factors it was conjectured from theoretical considerations that the alcoholic extraction would also remove these two factors. Experiments instituted with that purpose have indeed shown that the extract contained the antineuritic and to a smaller extent the antiscorbutic factor. These two principles were however not present in the extract in such high proportions as the fat-soluble factor.

EXPERIMENTAL.

Fresh green cabbage was thoroughly minced and triturated with sand. Absolute alcohol was then added (500 cc. of absolute alcohol for every 100 g. of cabbage) and the mixture allowed to digest in a cool dark place for about 12–18 hours. It was then filtered through a fluted filter and the residue on the filter paper pressed out and filtered. The combined filtrates were green and transparent and possessed the usual appearance of an alcoholic solution of chlorophyll. The alcoholic extract was then evaporated *in vacuo* at 35°. As the solution became concentrated chlorophyll separated out leaving behind a light brown liquid. The addition of alcohol to this solution which was practically free from alcohol produced a small white precipitate which consisted most probably of protein. On further concentration a greenish brown sweet syrup containing reducing sugars was obtained. This syrup possessed the characteristic taste of boiled cabbage. An equivalent of 25 g. of fresh cabbage was added daily to a diet which was complete in every respect but which lacked the fat-soluble factor. The composition of this BC diet was described in a previous communication [Zilva, 1919]. Of the various rats fed on this diet only few consumed the food well enough to demonstrate the growth-promoting power of the extract. Fig. 1 gives the weight curves of two representative rats. The male animal consumed the best part of his food, on the average about 18 g. per day, with the result that it grew well and reached a satisfactory weight within the 11 weeks during which it was kept on the diet. The female animal consumed only about 10–12 g. of the food per day. Such an amount is hardly quantitatively adequate to promote normal growth even if the diet were qualitatively well balanced. It will be seen that although the animal grew, the rate of growth was below the normal. The experiments with the cabbage extract, however, served as a

preliminary indication and demonstrated that it was possible to extract the fat-soluble factor by the method described.

In order to obviate the irregularity in the food intake of the rats carrots were next employed as a source for the accessory factor. These vegetables were treated in the same way as the cabbage. The filtrate obtained in this case was brilliantly clear and yellow in colour. On concentration *in vacuo* at 35° a very sweet and pleasant-tasting syrup was obtained. The syrup could be dissolved in water producing a turbid reddish yellow solution. An equivalent of 25 g. of fresh carrots was given to the animals in their daily diet at first. This preparation the animals consumed very well and as will be seen from Figs. 2 and 3 they developed well on it. Fig. 2 represents the

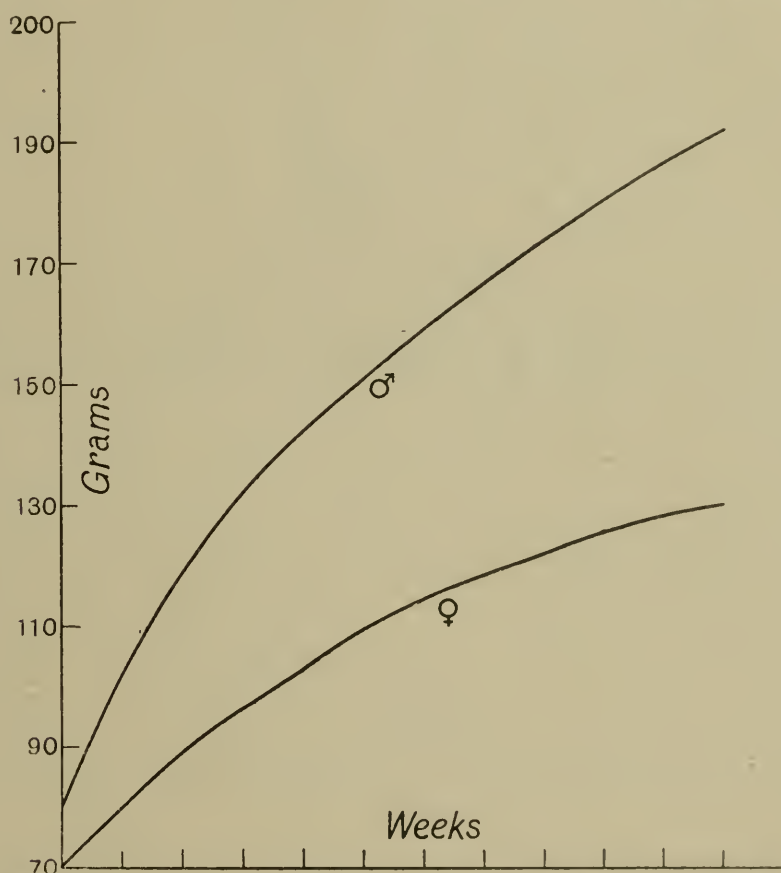


Fig. 1. Weight curve of rats fed on a diet containing the fat-soluble factor in the form of an alcoholic extract of cabbage.

weight curves of three animals which received the extract after they had subsisted for three weeks on a diet lacking the fat-soluble factor. They immediately resumed normal growth instead of ceasing to grow as was the case with the control animal. Fig. 3 shows how, on administration of an equivalent of 25 g. of fresh carrots to an animal which had existed on a diet free from the fat-soluble accessory factor for ten weeks and which was rapidly losing in weight, normal growth was induced.

It was next desirable to ascertain the minimum dose of the extract that would induce normal growth. Doses equivalent to 15 g., 5 g., 1 g. of fresh

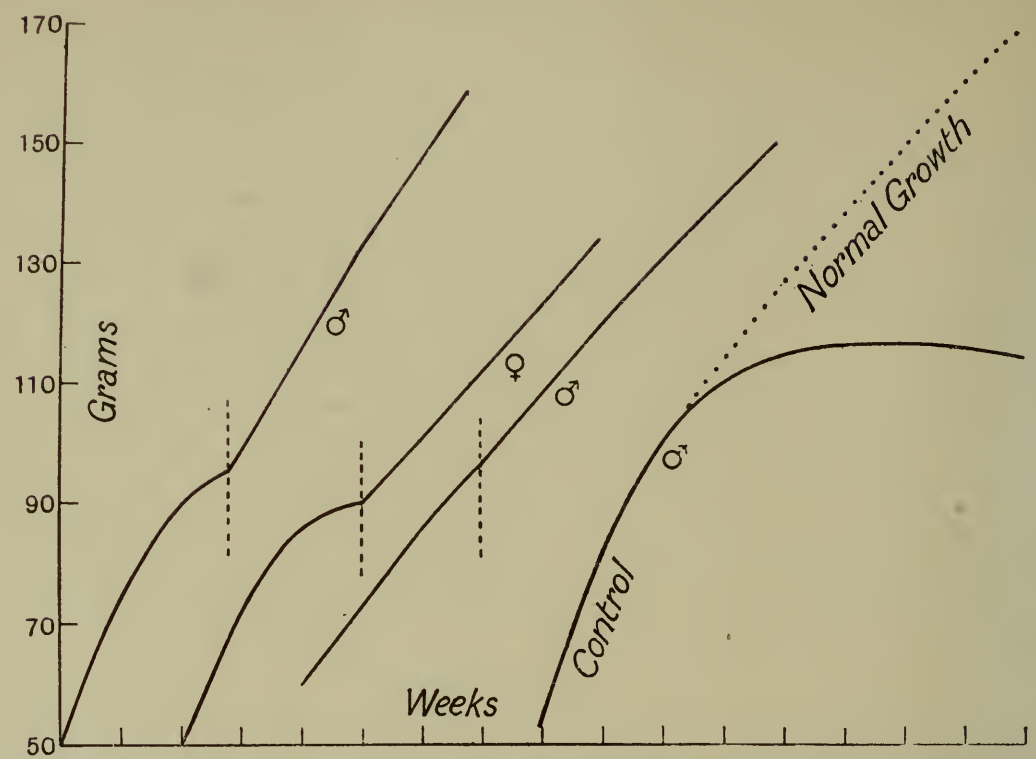


Fig. 2. Weight curve of rats fed on a diet containing the fat-soluble factor in the form of an alcoholic extract from carrots. The perpendicular dotted lines denote the commencement of the treatment.

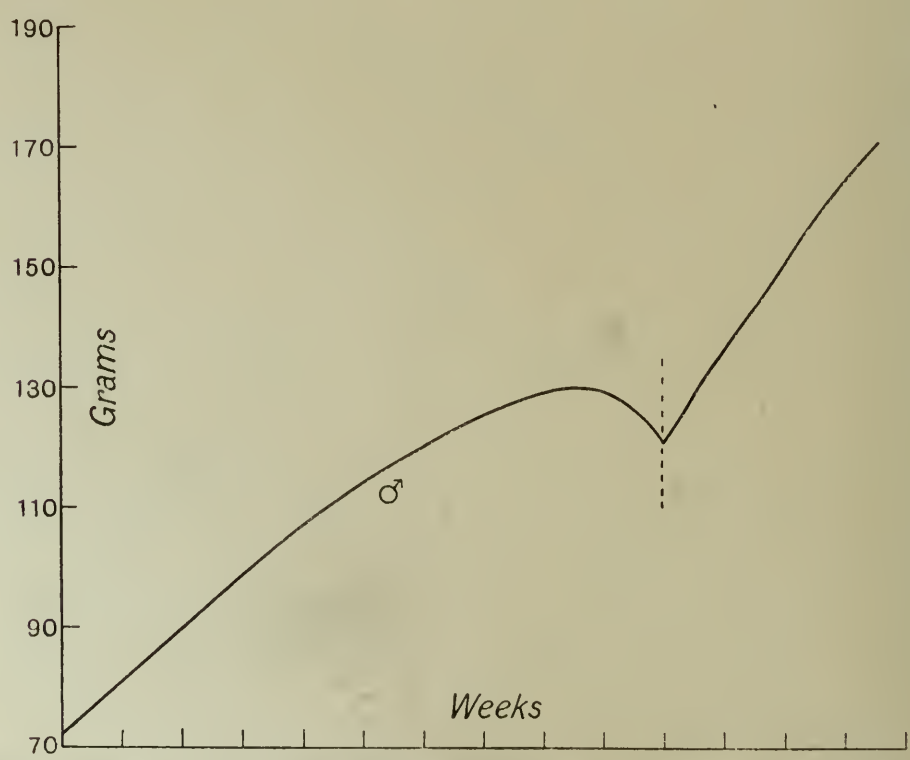


Fig. 3. The perpendicular dotted line denotes the commencement of the treatment.

carrots were given to three rats. In every case growth was induced (Fig. 4). The addition of the equivalent of 15 g. of carrots produced almost normal growth. The extracts from 5 g. and 1 g. produced less intense growth. An animal which had ceased growing for several months owing to the deficiency of the fat-soluble factor also resumed growth on receiving a dose equivalent to 1 g. of carrot. The growth curve in this case commenced flattening out after about ten days. The diet containing the 1 g. was consumed entirely. About 80 %–90 % of the other two doses was consumed daily by the rats. It may be pointed out in conjunction with the above figures that Steenbock and Gross [1919] found that 5 % of dried carrot was insufficient to produce normal growth while a diet containing 15 % sufficed for the purpose.

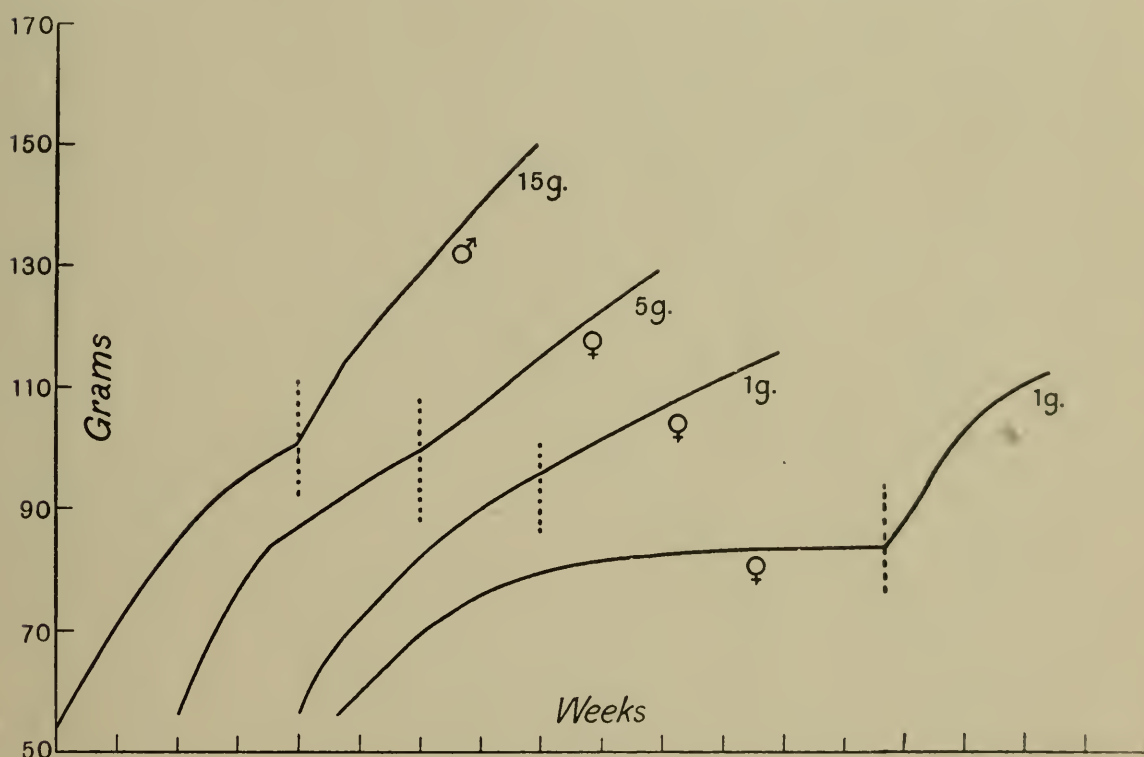


Fig. 4. The perpendicular dotted lines denote the commencement of the treatment.

Absolute alcohol extracts in the form of the syrup about 7.5 % of dry matter from fresh carrots which comprises about one-half of the total solids. On drying the syrup *in vacuo* at a low temperature and keeping the residue in a desiccator it was found that after a fortnight an equivalent of 25 g. of carrots was active both in inducing good growth and in curing xerophthalmia in rats. Any further particulars of the keeping properties of the carrot extract in dry condition have not yet been worked out.

The antineuritic factor was tested out on rats. Equivalents of 25 g. per day were added to an AC diet [Zilva, 1919]. It will be seen from Fig. 5 that this dose was sufficient to induce development a little below the normal rate. As these animals only consumed about 70 % of their daily diet it may be assumed that the dose was about the minimum necessary for normal growth.

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Fig. 5 also shows the weight curve of a rat which was deteriorating on account of an antineuritic deficiency but which on receiving the extract equivalent to 25 g. of fresh carrots resumed growth.

The tests for the antiscorbutic factor showed that the content of this principle in the carrot extract was very low. Three guinea-pigs kept on a diet of oats and bran and a daily ration of 40 cc. of autoclaved whole milk received after the tenth day a daily dose of the extract equivalent to 25 g., 10 g. and 5 g. of fresh carrots respectively. The animals which received the 10 g. and 5 g. doses developed scurvy, declined in weight and eventually died in

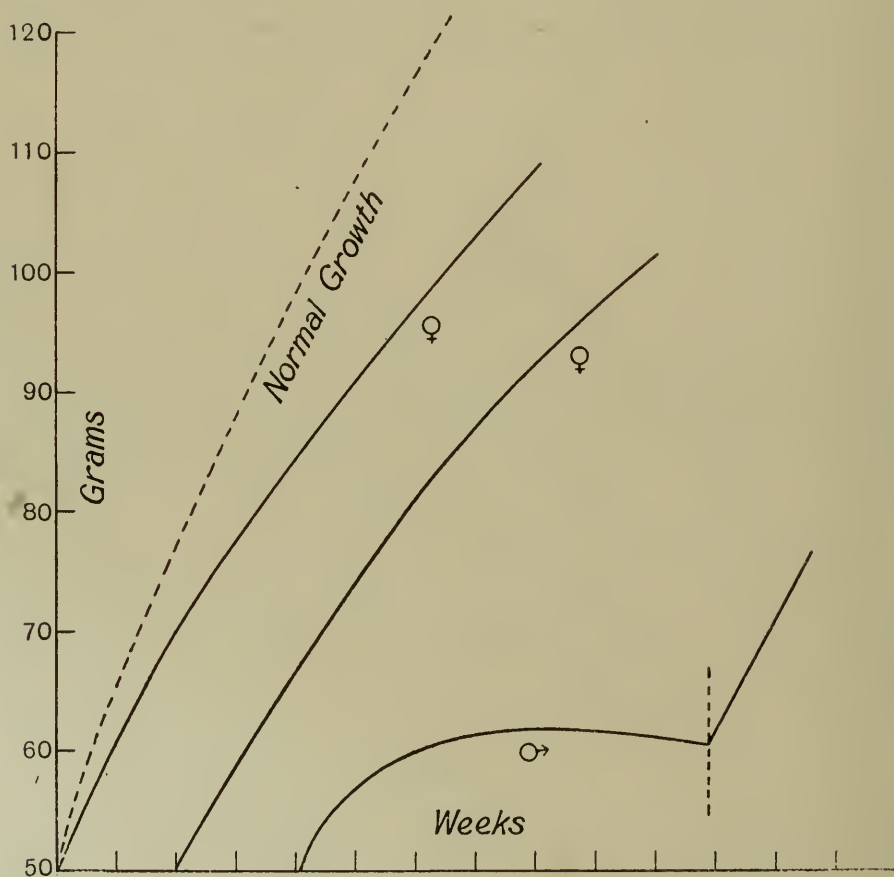


Fig. 5. The perpendicular dotted line denotes the commencement of the treatment.

the ordinary way as they would have done on a purely scorbutic diet (Fig. 6). No delay in the onset of the symptoms or in the fatal termination could be recorded. The guinea-pig which received daily the extract from 25 g. of fresh carrots showed a decided delay in the onset of the scorbutic symptoms. Unfortunately this animal succumbed to some other complaint on the 23rd day. At the post mortem examination mild signs of scurvy were observed which were not acute enough to be responsible for the death of the animal. The extract from 25 g. of carrots contains therefore enough antiscorbutic to delay the onset of the disease¹.

¹ [Note added on 9th of June.] A daily administration of an alcoholic extract equivalent to 50 g. of fresh carrots protected a guinea-pig subsisting on a scorbutic diet for 42 days.

The alcoholic extract from carrots was next concentrated until all the alcohol was driven off and the aqueous solution became concentrated. This solution was extracted by shaking it with ether in a separating funnel. The ethereal solution at first was yellow but on repeated extraction no more colouring matter could be removed from the solution which nevertheless still retained a fairly intense yellow colour. A solution equivalent to 250 g. of fresh carrots extracted with ether as described yielded a residue weighing 0.4935 g. The residue, which was dark brown, on drying for two hours at 110° remained of oily consistency. On extracting it with sodium carbonate and acidifying the solution no precipitate was obtained which would indicate that it did not contain any free higher fatty acids. The residue was next saponified by heating with alcoholic potash under a reflux condenser for two hours, the alcohol driven off, and the aqueous solution filtered. On adding acid to the filtrate a solid substance resembling the higher fatty acids separated out and gradually accumulated on the surface of the liquid. There is little doubt then that the ethereal extract contained some oil.

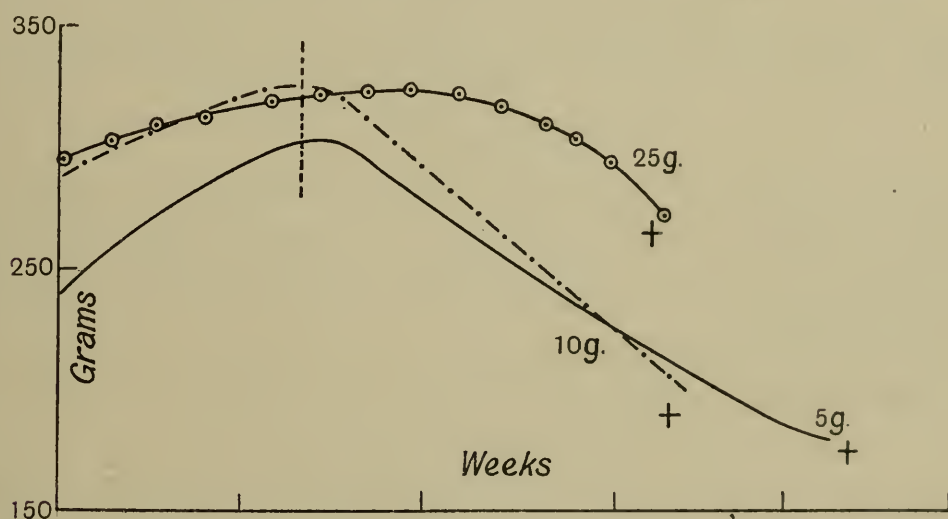


Fig. 6. The perpendicular dotted line denotes the commencement of the treatment.

In order to test the activity of this fraction autoclaved olive oil was added to the ethereal solution and the ether evaporated. A highly coloured and opaque oil was left behind. Both the colour and the opacity were dependent on the strength of the extract. Through the medium of the oil the ethereal extract, equivalent to 25 g. of carrots, was incorporated in the daily diet which was free from the fat-soluble factor and as will be seen from Fig. 7, which represents the weight curve of two rats treated with this fraction, growth was promoted immediately the treatment was commenced.

While these experiments were in progress Osborne and Mendel [1920] published a short note in which they state that they have extracted with ether from spinach leaves and young clover dried at 60° an oily residue which was active in small quantities. Although they have obtained their fraction from different plants and directly from the dried vegetable their work corroborates the fact that the fat-soluble factor in plants is soluble in ether. Further

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experiments with various solvents carried out preferably on the alcoholic fraction ought to throw some light on the subject whether the activity of the ethereal fraction is due to the small amount of the oil or to some other substance associated with it.

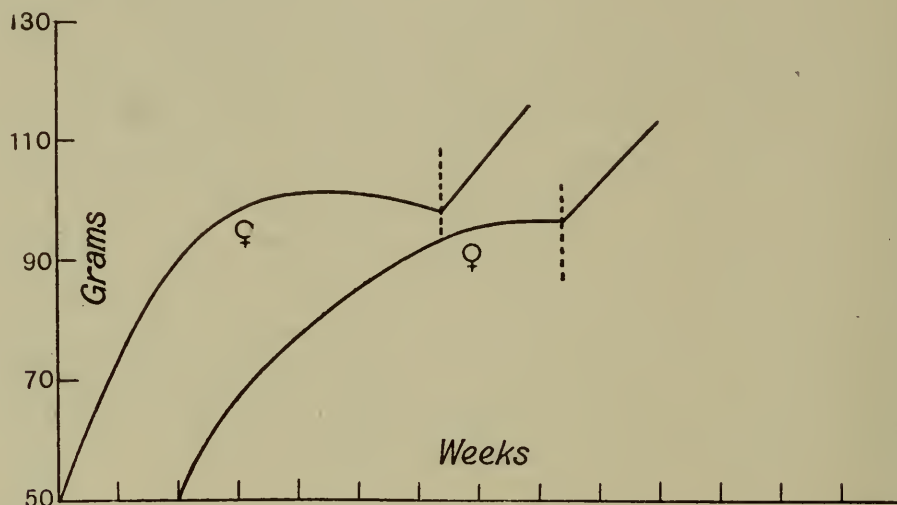


Fig. 7. The perpendicular dotted lines denote the commencement of the treatment.

SUMMARY.

1. Alcohol extracts the fat-soluble factor from cabbage and carrots.
2. The alcoholic extract equivalent to 10–12 g. of fresh carrots given daily is sufficient to promote normal growth in rats subsisting on a diet lacking the fat-soluble factor.
3. The alcoholic extract from carrots also contains the antineuritic and to a smaller extent the antiscorbutic factors.
4. An ethereal extract from the alcoholic fraction equivalent to 25 g. of fresh carrots has been found to promote recovery and renew growth in rats declining in weight on account of a fat-soluble factor deficiency.

Part of the expense of this research was defrayed from a grant made by the Medical Research Council, to whom my thanks are due.

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Nº 17

LXXI. THE ACTION OF OZONE ON THE FAT-SOLUBLE FACTOR IN FATS.

PRELIMINARY NOTE.

BY SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute.

(Received October 18th, 1920.)

IN a previous communication [Zilva, 1919] it was shown that when butter was spread in a thin layer on a plate and exposed to ultra-violet rays for six to eight hours, the fat-soluble factor in it was inactivated. It was then pointed out that as ozone was produced by the mercury quartz lamp from the atmospheric oxygen the butter was at the same time exposed to the action of this gas, and that further investigation would be required in order to ascertain whether the inactivation was due to the action of the rays or to that of the ozone. It was therefore decided to establish the influence of ultra-violet rays on the fat-soluble factor in the absence of oxygen and the action of ozone on it in the dark, as well as to determine the iodine value of the fat under different conditions of exposure. As the results were of great interest, the inquiry into the action of ozone was extended to the antineuritic and antiscorbutic factors. The object of this note is to give a brief summary of the results obtained by studying the action of ozone on the fat-soluble factor only. The experimental data are reserved for another communication in which the influence of ozone on all the accessory factors will be described and discussed in detail.

For technical convenience active fats which are transparent and liquid at room temperature, like whale oil and cod liver oil, were chosen for this investigation. The latter oil being very active was found to be the more suitable and most of the experiments were therefore carried out with it. On exposure for six to eight hours in shallow layers in Petri dishes to the action of the ultra-violet rays in an atmosphere containing ozone these oils were entirely inactivated.

Unlike the butter, however, the cod liver oil was not bleached but assumed a slightly darker colour, which suggests that the nature at least of some of the colouring matter associated with it is different from that of butter.

In order to study the effect of ultra-violet rays on the fat-soluble factor in cod liver oil in the absence of oxygen the oil was placed in a thin layer a few millimetres thick between two tubes, one of which fitted loosely into the

other. The outer tube was made of quartz, so that the ultra-violet rays could reach the oil without being previously absorbed. The air above the layer of oil was displaced by carbon dioxide gas, and the tube was revolved by a water motor during the exposure. On testing the exposed oil on rats receiving a diet deficient in the fat-soluble factor it was found that an exposure even of 16 hours' duration did not inactivate the oil, nor was there any evidence that the activity of the oil was impaired to any great extent by such an exposure.

The next set of experiments was carried out with cod liver oil exposed to ozone in the dark. This was done by introducing some of the oil into a dark-stained glass bottle through which a current of ozone was passed. By rolling the bottle at short intervals the oil was thoroughly exposed. About ten hours of this treatment almost solidified the oil at the concentration used. After six hours' exposure the oil was much more viscous than before treatment, and high doses of this modified oil, which was originally extremely active, failed to promote growth in rats deficient in the fat-soluble factor.

It is evident then that ozone inactivates the fat-soluble factor in active oils and fats. This is in agreement with the recent observations of Hopkins [1920, 1, 2] and Drummond and Coward [1920] that the fat-soluble factor in fats on being exposed to atmospheric oxygen becomes inactivated. The action of ozone is of course much more drastic and therefore more rapid.

I wish to express my indebtedness to Dr J. S. Edkins for having kindly permitted me to use his ozone generator.

A part of the expenses of this research was defrayed from a grant made by the Medical Research Council to whom my thanks are due.

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Nº 18

THE INFLUENCE OF AERATION ON THE STABILITY OF THE ANTISCORBUTIC FACTOR.

BY S. S. ZILVA, D.SC., PH.D., F.I.C.,

ASSISTANT, BIOCHEMICAL DEPARTMENT, LISTER INSTITUTE.

THIS preliminary note describes the results obtained from some experiments instituted with the object of studying the influence of air on the antiscorbutic factor at ordinary temperature as well as at 100°C. The inquiry was pursued as an outcome from observations made that ozone inactivated this and the fat-soluble factors at ordinary temperature. The experiments revealed that bubbling air at ordinary temperature through an antiscorbutic solution inactivated it. It was found, on the other hand, that on boiling an active solution for two hours in an atmosphere of CO₂ no marked diminution in the activity could be recorded, whilst on boiling a similar solution for one hour during which time air was bubbled through almost the entire activity was lost.

These results are in conformity with the observations made by Delf (1920).¹ Miss Delf found that quite marked antiscorbutic potency was retained by orange-juice and swede-juice heated for some time at temperatures above 100°C., and she suggested that the destruction may have been affected directly either by retarded oxidation (the juice having been heated in an autoclave) or indirectly by the production of stabilising bodies. My experiments seem to point to the fact that the former is the more likely explanation for this somewhat unexpected stability observed by her in the case of the juices.

As a source for the antiscorbutic factor lemon-juice from which the organic acids were removed by precipitation with calcium carbonate was employed.

¹ Delf (1920), *Biochem. Journ.*, xiv., 211.

The minimum dose of this solution for the guinea-pig is about $1\frac{1}{2}$ –2 c.cm. When air was aspirated through such a solution for 12 hours at laboratory temperature daily doses of 3 c.cm. and 5 c.cm. were insufficient to prevent, but delayed very slightly, scurvy in guinea-pigs, whilst 7 c.cm. was found to be inadequate to promote normal growth. Daily doses of $1\frac{1}{2}$ c.cm., 3 c.cm., and 6 c.cm. of the decitrated juice which was boiled for one hour and through which air was bubbled during the boiling were insufficient to prevent or to delay to any very marked degree the onset of scurvy in the animals. From the administration of daily doses of 1.5 c.cm., 4 c.cm., and 6 c.cm. of the juice boiled in an atmosphere of CO₂ for two hours no inactivation which would become manifest by our present technique could be established.

It is of interest to note that the behaviour of the antiscorbutic factor towards heat and aeration is very similar to that of the fat-soluble factor. The antineuritic factor, however, seems to be more stable towards oxidation. Results obtained by exposing the antineuritic factor to ozone show that it is only affected, if at all, to an extremely small extent by that treatment.

All the data available in connexion with the behaviour of the antiscorbutic and the fat-soluble factors at high temperatures will have to be reconsidered in view of the latest results obtained as regards their great tendency to be inactivated on aeration, especially when heated.

I am indebted to Professor Sensho Hata for help in some of the experiments of this inquiry.

The expenses of this research were defrayed from a grant made by the Medical Research Council, to whom my thanks are due.

XLIX. THE RELATION OF THE FAT-SOLUBLE FACTOR TO RICKETS AND GROWTH IN PIGS.

BY SYLVESTER SOLOMON ZILVA, JOHN GOLDING,
JACK CECIL DRUMMOND AND KATHARINE HOPE COWARD¹

*From the Biochemical Department, Lister Institute, London; the Research
Institute in Dairying, Reading; and the Institute of Physiology,
University College, London.*

(Received May 3rd, 1921.)

CLINICAL and experimental observations tend to show that diet plays an important part in the etiology of rickets and much of the evidence obtained by the clinician goes further to demonstrate that the results obtained with certain oils and fats in the prophylactic and the curative treatment of the disease is quite marked. The recent experimental work of Mellanby [1918 and 1919] throws a very interesting light on the therapeutic value of fats and oils. Mellanby has shown that by keeping puppies on a certain basal diet, definite rickets could be induced in these animals and that the disease could be prevented or cured by the addition of certain substances, mostly oils and fats, to the basal diet. Of the substances which were capable of producing this beneficial effect the majority were found to be identical with those which contain the fat-soluble A factor. This important observation suggested the possibility that the fat-soluble factor or another accessory factor closely associated with it was concerned in the prophylaxis of rickets. However, investigations carried out clinically and experimentally in order to test this theory of the etiology of rickets have yielded results from which no definite conclusions can yet be drawn. Hess and Unger [1920] from observations made on groups of infants receiving diets some of which were rich, others deficient in the fat-soluble factor, could not obtain any definite evidence that the rickets which developed in some of their patients could be traced to an accessory factor deficiency. Harden and Zilva [1919] kept young monkeys on a diet deficient in the fat-soluble factor for several months. The animals declined, but no rickets developed. Mackay [1921] fed kittens on a diet deficient in this factor, but otherwise theoretically adequate, with the result that the animals ceased growing. No evidence of rickets however could be established at the post mortem examination. McCollum, Simmonds, Parsons, Shipley and Park [1921] recently analysed a series of diets on which rats developed rickets and although the

¹ Beit Memorial Research Fellow.

results pointed to the possibility that a deficiency of the fat-soluble factor or calcium might be responsible for the production of the disease, the authors considered that with the experimental evidence so far at their disposal they were only justified in concluding that faulty nutrition was the cause of the observed rachitic changes.

We are at present engaged in studying various dietetic problems including the etiology of rickets in the pig. The pig is a very suitable animal for such a purpose as it is susceptible to rickets, is omnivorous and can be reared away from its mother on experimental diets.

The cause of rickets in the pig has often been attributed by agriculturists to a dietetic deficiency—calcium and phosphorus being the suspected elements. Thus one finds statements in various text-books on scientific feeding that pigs fed on potatoes, whey, maize and cereals develop rickets. If on the other hand such diets are supplemented by clover or meadow hay the disease does not occur. Although calcium is usually considered to be the limiting factor it will be noticed that the diets which are alleged to produce rickets in the pig are at the same time deficient in the fat-soluble factor, whilst clover and meadow hay besides supplying calcium also form a source for this accessory factor.

Herter [1898] on the assumption that fat deficiency might be responsible for the production of rickets placed young pigs two months old on a diet of skimmed cow's milk. The animals deteriorated after several months but in the author's opinion rickets did not develop. It is, however, of interest to note that Herter found that phosphates were imperfectly absorbed when this diet low in fat was used. The diets were of course also low in the fat-soluble factor.

Our primary object was to find out whether pigs, brought up from birth on a diet rigorously restricted in the fat-soluble factor only, would develop rickets with the regularity with which animals, which are susceptible to scurvy, develop this disease on a scorbutic diet. Owing to the exacting attention that such experiments require we could only deal at first with a limited number of animals. The results obtained concerning the original object in view were not definite, but other observations of interest were made which will be discussed in this communication.

EXPERIMENTAL.

The experiments were carried out at the Reading University College Farm, Shinfield. Four animals, *A*, *B*, *C* and *D*, which were divided into two groups, were employed in this experiment. Group I, consisting of *A* and *B*, were placed on a diet containing the fat-soluble factor, whilst Group II, consisting of *C* and *D*, were kept on a diet rigorously restricted in that factor. The animals, which weighed three pounds each at the commencement of the experiment, were young Berkshire boars farrowed on August 13th out of "Whitley Sensation" by second prize boar "Swinton Cogniac." On August 17th they were placed on the experimental diets. During the first three days a mixture resembling

in composition sow's colostrum was given to the animals. The diets were of the following composition:

<i>Group I.</i>		<i>Group II.</i>	
Extracted dried milk	7.75 g.	Extracted dried milk	7.75 g.
Cream (cow's milk)	20.2 „	Autoclaved olive oil	9.5 „
Purified caseinogen	12.6 „	Purified caseinogen	13.0 „
Salt mixture	0.28 „	Salt mixture	0.36 „

The above was made up to 100 cc.

On August 20th the above diets were changed to mixtures approximating in composition to sow's milk, which were made up according to the following formulae:

<i>Group I.</i>		<i>Group II.</i>	
Fresh cow's milk	60 cc.	Extracted dried milk	6.7 g.
Crude caseinogen	5.0 g.	Purified caseinogen	4.9 „
Cream (cow's milk)	5.0 „	Autoclaved olive oil	4.6 „
Salt mixture	0.66 „	Salt mixture	0.63 „

The above was made up to 100 cc.

To insure the solution of the caseinogen 5 % sodium bicarbonate (20 cc. per 100 g. of caseinogen) was used.

Daily doses of decitrated lemon juice and "marmite" were administered to supply the antiscorbutic and the antineuritic requirements of the animals.

The dried separated milk was extracted with light petroleum for two to three days.

The cream was freshly separated and was obtained daily from the Reading College Dairy. Its average fat content, as determined by the Gerber method, was 42 %.

The caseinogen was rendered free from detectable traces of the fat-soluble factor by being heated for 24 hours in shallow layers at 120° C. and being subsequently extracted for one day with 90 % alcohol and for two days with light petroleum. The olive oil was autoclaved for $\frac{1}{2}$ hour at a pressure of two atmospheres. The salt mixture was of the same composition as that employed by McCollum and his collaborators.

As will be seen, the chief difference between the diets in the two groups was in the content of the fat-soluble factor. In Group II the active cream was replaced by the inactive olive oil.

At the commencement of the experiment the little pigs were fed by bottle every two hours day and night. After August 23rd the intervals were extended to three hours. By the end of August the animals received six meals a day, which were further reduced by September 15th to five meals, with a night interval of six hours. The diets were given *ad lib.* and the quantities consumed were recorded.

At first the intake of food and the growth of the two groups were approximately the same. After a few days, however, the animals in Group II commenced consuming less food than the animals in Group I and at the same

time ceased growing, whilst the control animals continued to gain in weight. See Photograph 2, Plate II.

On September 14th pig *D* died during the night. A skiagraph was taken soon after death, but no abnormalities could be discerned. At the post mortem examination very pronounced "beading" at the costo-chondral junctions of the ribs was observed. Photograph 1, Plate II, shows the appearance of the costo-chondral junctions of the ribs of this animal. No other abnormal changes were established. The analysis of one of the long bones gave the following figures: moisture, 20.1 %; CaO, 29.3 % of the dry weight. We have so far been unable to obtain a really normal animal of the same age for comparative purposes. Histological preparations of the ribs, which will be discussed later, were kindly made for us by Miss F. M. Tozer, to whom we take this opportunity of expressing our best thanks.

In order to save the other declining animal in this group, cream was added to its diet in the same proportion as that in the diet of Group I. It was intended to supply a small amount of the fat-soluble factor sufficient to stop further decline. The animal soon resumed growth and the cream was accordingly discontinued on September 27th. From September 14th this animal received, besides the artificial milk mixture, an addendum of a basal mixture made up of 150 parts of starch, 40 parts of purified caseinogen and 10 parts of the salt mixture. After the cream was discontinued crude caseinogen was employed in this mixture instead of the purified substance in order to supply a further very limited amount of the fat-soluble factor. An average of about 250 g. of this mixture was consumed by the animal per day. From October 16th purified caseinogen was again employed, and on October 26th the artificial milk was replaced by a mixture consisting of olive oil, extracted dried milk, basal mixture and salt mixture. The olive oil and the caseinogen were discontinued on November 22nd. At this period the animal was consuming about 2 lb. of starch and $1\frac{3}{4}$ lb. of extracted fat-free dried milk per day. Towards the end of the experiment, owing to a temporary shortage of extracted dried milk, a mixture of starch and purified caseinogen was again resorted to.

As will be seen from Fig. 1, which represents the weight curves of the animals, pig *C*, in spite of the restricted diet, kept on growing after the resumption of growth produced by the administration of the cream even better than the animals in the control group. No symptoms of rickets were observed during the experimental period. A skiagraph was taken on November 9th and a normal picture was obtained. The animal was slaughtered on January 19th. At the post mortem examination the following was found: Skin—good and clean. Thyroid glands—more connective tissue than in the control animal. Femur decidedly thicker and distinct enlargement of all bones at knee joint. Tarsal bones thicker than in control. Junctions between tarsals and metatarsals also thicker than in control. Spleen—a little heavier than in control. Liver—superficial foci of inflammation; weight of liver, $2\frac{1}{4}$ lb. More fat was found in this animal than in the control. Kidney normal. Adrenals



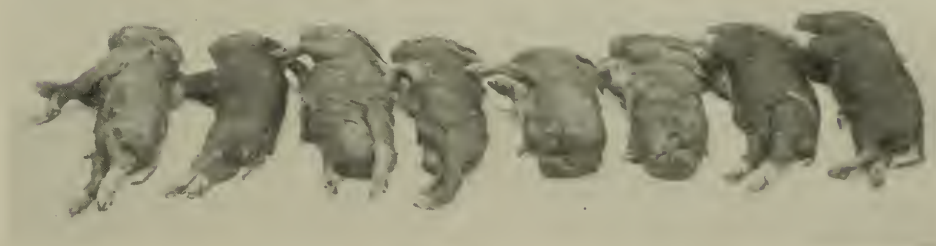
Photograph 1. Ribs from *D*.



Photograph 2. *A* and *C* 28 days old.



Photograph 3. *C* before being slaughtered.



Photograph 4. Litter from Sow Lot I.



Photograph 5. Litter from Sow Lot I.

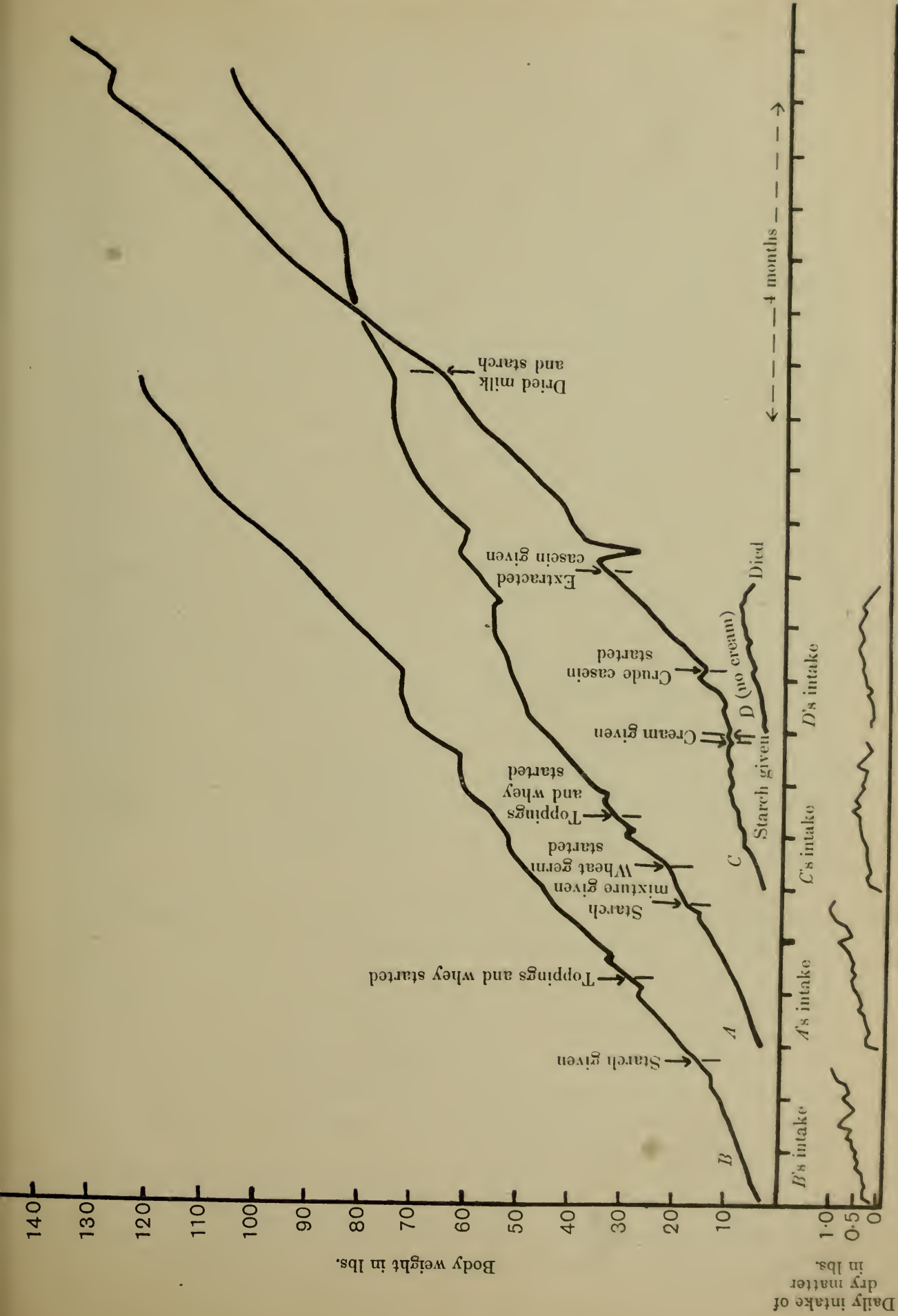


Fig. 1

normal. Ribs—angle of junction between bones and cartilage at costo-chondral junction markedly greater than in the control. In attempting to cut with a knife the same resistance was met as in the case of the control. The cartilage at the costo-chondral junctions strikingly thinner than the bony part as compared with the control animal. Longitudinal section at costo-chondral junction is decidedly wider than in the control and the line of active cartilage is also a little wider. Heart and lungs quite normal. Weight of leaf fat, 1 lb. 14½ oz. The analysis of one of the long bones gave the following figures: moisture, 14.7 %; CaO, 37.6 % of the dry weight. Photograph 3, Plate II, is a picture of pig *C* before being slaughtered.

In the control group pigs *A* and *B* commenced showing weakness in their legs, animal *A* in the hind and animal *B* in the front limbs and afterwards also in the hind legs. The wooden floor was at this stage replaced by bricks and the animals somewhat improved soon after the change. This affection of the limbs was definitely established to be non-rachitic. On September 13th an addendum of basal mixture and wheat germ was made to both diets. From September 20th lucerne was given to pig *B* and from October 1st about 15 g. of crude cod-liver oil was administered to pig *A*. On October 6th the milk was discontinued in both cases, pig *A* receiving basal mixture, wheat germ, toppings and whey; pig *B* receiving crude caseinogen, toppings and whey. From November 22nd the diet of pig *A* consisted of toppings, whey and cod-liver oil, which was supplemented by maize gluten on December 23rd. The diet of pig *B* was supplemented by maize gluten on December 15th. Pig *B* was killed on January 19th and pig *A* on February 17th. At the post mortem examination no rickets could be established in either case. A piece of wire was found to have penetrated the stomach of pig *A* and to have caused local inflammation which most probably was responsible for the ill-health of the animal during the last few weeks of the experiment. The analyses of the long bones gave the following figures: pig *A*, moisture 16.8 %, CaO 35.5 % of dry weight.

On three occasions during the early part of the experiment the weights of our experimental animals were compared with those of the little pigs of the same litter which remained with the mother. On September 8th the pigs in the litter weighed 16½, 15, 13, 14½, 14¼ lb. whilst pig *A* weighed 14 lb., pig *B* 12¾ lb., pig *C* 8 lb. and pig *D* 9¾ lb. On September 24th the pigs in the litter weighed 21¾, 23¾, 26¼, 18½ lb., and pig *A* weighed 25½, pig *B* 22¾, pig *C* 12¼ lb. On November 26th the average weight of the litter pigs was 54.7 lb., whilst pig *A* weighed 65¼, *B* 72¼ and *C* 72 lb. It is therefore evident that the pigs brought up artificially gained more weight than the animals which remained with the mother.

The ribs of pigs *A*, *C* and *D* were examined histologically and the observations may be summed up as follows:

(1) A moderate increase in the number of proliferating and hypertrophic cartilage cells was found only in pig *C*.

(2) A deep penetration of blood vessels from the bone marrow into the cartilage was observed in all the three cases.

(3) The presence of fibro-cellular tissue instead of normal marrow was found in all cases. It was more pronounced in the case of pig *C*.

(4) The irregularity of the line at the costo-chondral junction was also recorded. This was most marked in the case of pig *D*.

The back and leaf (perinephritic) fats from pigs *A*, *B* and *C* were tested by our standardised technique for the presence of the fat-soluble factor on rats, and as will be seen from Fig. 2 which represents the weight curves of these rats, the fats derived from animals fed on a diet containing this factor were found to be more or less active; the reverse was the case with the fats from the animal fed on a deficient diet. There was one discrepancy in the case of the leaf fat of pig *B* which appears to have been due to an irregularity in the technique; unfortunately we did not have enough of this sample to repeat the test. These observations confirm the results we [1920] have previously obtained with pigs fed on diets of varying contents of the fat-soluble factor.

No marked difference was found in the water, connective tissue and nitrogen contents of the two fats. The refractive indices, the Polenske, iodine and potash absorption values were also almost identical.

The results of our experiments offer no definite information on the relation of the fat-soluble factor to the etiology of rickets. We are however of the opinion that if the deficiency of this factor alone bore the same relation to the etiology of rickets as that of the antiscorbutic and antineuritic factors to the etiology of scurvy and beri-beri, we should have obtained a better differentiation in the rachitic condition in the two groups, in spite of the limited number of animals employed. As it is, we find it impossible with the results so far at our disposal to explain the decided, although slight, rachitic changes observed histologically in our normally-fed animals.

The above experiments, however, bring into prominence the following points:

(a) That when pigs are "off their feet" it does not necessarily imply that it is due to rickets. The affection of the limbs of pigs *A* and *B* was decidedly not of a rachitic nature.

(b) That the requirements of the pig for the fat-soluble factor are of a low order. This has already been pointed out by us [1920] in a previous communication. In this investigation the comparatively small quantity of cream and crude caseinogen consumed by pig *C* was sufficient to act as a source of the fat-soluble factor for the animal during about four months.

(c) That the fat-soluble factor promotes growth in the pig. This was demonstrated by the resumption of growth of pig *C* on the addition of cream to the restricted diet. It was further confirmed by the following experiment, which is a continuation of that described in the previous communication already referred to and in which we kept pigs on diets consisting of toppings

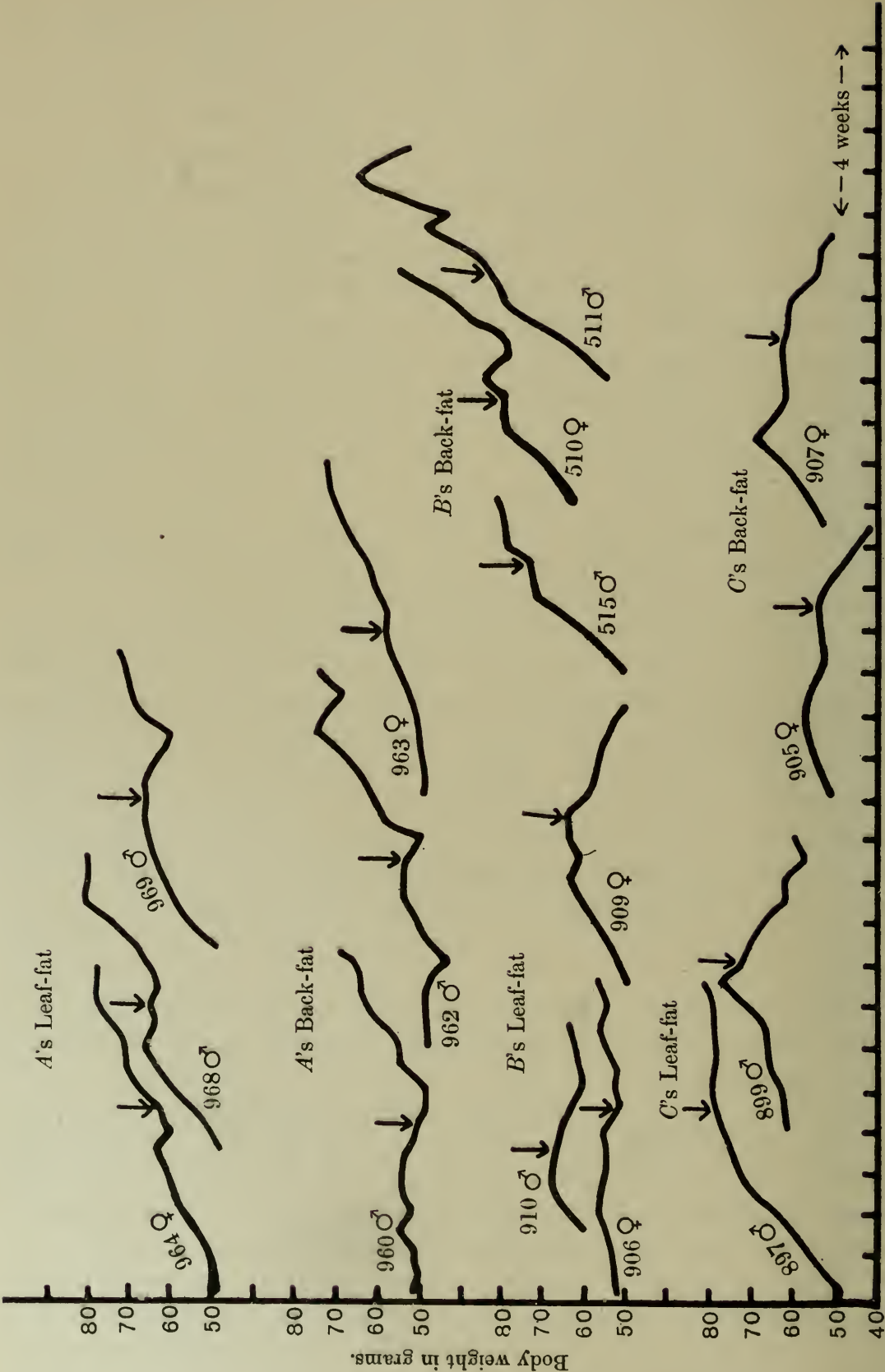


Fig. 2

and synthetic whey (Lot I) and toppings and ordinary whey (Lot II). Both these diets were highly deficient in the fat-soluble factor, the diet of Lot I being the more restricted. In the early stages of the experiments these animals showed a considerable growth which eventually slackened off. On September 23rd the sows belonging to Groups I and II had their restricted diets supplemented by an addendum of the fat-soluble factor. At this time the animal in Lot I had practically ceased growing, whilst the animal in Lot II was growing at a rate much below the normal. It was decided to administer the fat-soluble factor to the sow in Lot I in the form of crude cod-liver oil which was found to be very active in the case of rats and to the sow in Lot II in the form of lucerne. Lot I was given at first $\frac{1}{4}$ oz. of cod-liver oil per day; this was after five days increased to $\frac{1}{2}$ oz., and after 42 days this was again increased to 1 oz. per day for 20 days. The animal consumed the oil with great alacrity and resumed growth immediately after the commencement of the treatment. Lot II was started at first on $\frac{1}{2}$ lb. of lucerne per day; this after five days was increased to 1 lb., and after two weeks to 4 lb. per day. On December 3rd both sows were taken to the boar and became pregnant. They were then placed again on their original restricted diets. Fig. 3 represents the weight curves of these animals as well as that of the sow belonging to Lot III, which was fed all the time on toppings, whey and grass, a diet rich in the fat-soluble factor. On March 30th the sow in Lot I gave birth to eight pigs all of which were born dead or died very soon after birth.

One of the young lived for five hours and on post mortem examination was found to be normal except for a slight general oedema. Of the remaining seven pigs four showed a marked abnormality in the development of the hind limbs to a varying degree. This malformation was particularly pronounced in two cases (see Photographs 4 and 5, Plate II) in which the hind limbs were represented by thin tail-like appendages.

Oedema was generally present in all the eight bodies to a varying degree; four cases showing very marked ascites, hydropericardium and hydrocele, but there was no association between the severity of the oedema and the malformation of the limbs. The large hydroceles in these cases may be seen from the photographs. In nearly all cases there was more or less severe hydro-nephrosis.

The weights of the eight young were respectively 950, 1100, 1250, 900, 950, 1050, 1100 and 1350 g.

Analyses of the bones of three of the pigs were made, but their value is limited by the fact that only one analysis from a normal pig of the same age was available.

	Pigs from litter Lot I.			Normal control
	1	2	3	
	g.	g.	g.	g.
Weight of humerus	5.2	5.68	4.27	4.5
% moisture	41.6	46.3	44.5	40.9
% CaO on dry weight	34.45	33.71	32.80	37.24

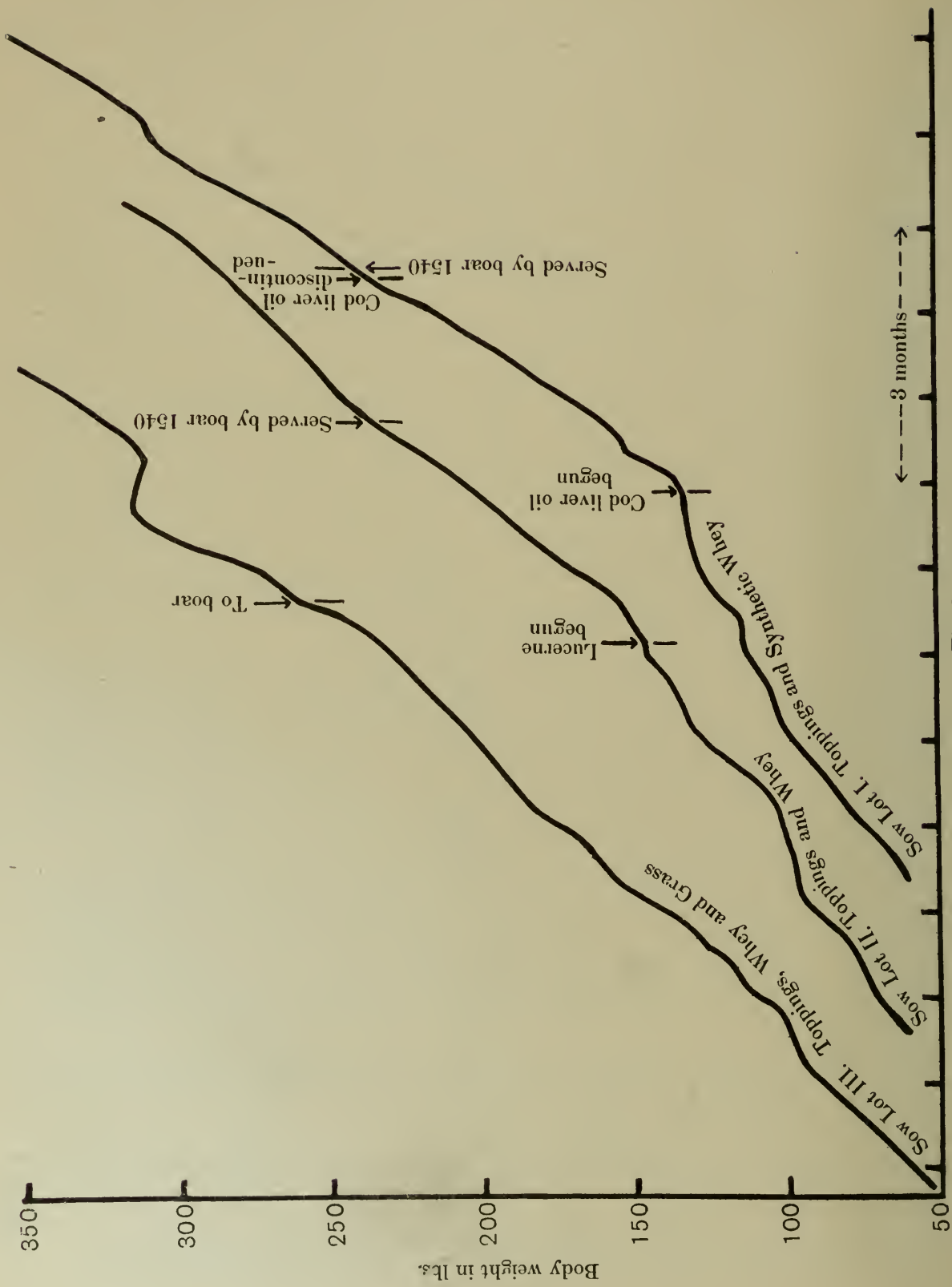


Fig. 3

We are not at present in a position to say whether this abnormal litter is to be ascribed to the drastically restricted diet which the mother had received, but further experiments are in progress.

The sow in Lot II farrowed down nine pigs on April 8th of which only one died.

In conclusion we wish to express our indebtedness to Mrs John Golding for the indefatigable help she rendered us during this inquiry.

The expenses of this research were defrayed from a grant made by the Medical Research Council, to whom our thanks are due.

SUMMARY.

No definite rickets was induced in sucking-pigs fed from birth on a diet rigorously restricted in the fat-soluble factor.

The addition of the fat-soluble factor in the form of cream, cod-liver oil, and lucerne to a deficient diet stimulated growth in pigs declining in weight.

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XLVIII. THE DIFFERENTIAL DIALYSIS OF THE ANTINEURITIC AND THE ANTISCORBUTIC FACTORS.

BY SYLVESTER SOLOMON ZILVA AND MASATARO MIURA.

From the Biochemical Department, Lister Institute.

(Received May 3rd, 1921.)

It has been shown by Eijkman [1906] that the antineuritic factor is dialysable. Holst and Frölich [1912] also found that when fresh cabbage juice was dialysed for 3-4 days it lost the best part of its activity although all the salts did not disappear from the juice during that time. It is therefore evident that under certain conditions the antineuritic and the antiscorbutic factors will pass through a dialysis membrane. As it is now possible to calibrate the permeability of collodion membranes we set out to ascertain what degree of permeability of collodion membranes would permit the passage of these factors. Brown [1915] has shown that if air-dried collodion membranes, which are highly impermeable, are placed in solutions of alcohol and water they become permeable, that the permeability increases with the strength of the alcohol employed and that the degree of permeability of such membranes can be established by various methods based on certain properties which display a parallelism with the permeability. Utilising the above principles we have determined the permeability of the collodion membranes which permit the passage of the antineuritic factor of autolysed yeast juice and the antiscorbutic factor of lemon juice from which the citric acid has been removed.

EXPERIMENTAL.

The solutions were dialysed through collodion thimbles. These were prepared in the following way: a 14 % alcohol-ether solution of collodion (356A/9) supplied by the Necol Industrial Collodion Ltd was introduced into a test-tube 14 cm. long and having an internal diameter of 3 cm. The collodion was then either centrifuged or allowed to stand for about 12 hours in order to permit the bubbles of air to rise to the surface, the opening of the tube being protected by an outside tube in order to obviate the evaporation of the solvents. The tubes were inverted and the collodion was allowed to drain for 5 minutes after which time the tube with the collodion was immersed in water and the thimble was stripped off. It was then washed in water, dried for 24 hours in

the air and soaked for 24 hours in the alcohol-water solution of the requisite strength. After washing the thimbles were kept in water until required for use.

40 cc. of the active solutions were dialysed against running water. The thimbles were closed with indiarubber stoppers through which passed a glass U-tube containing mercury.

In order to ascertain the permeability of our thimbles, substances of various molecular weights were allowed to diffuse through them and the time and the degree of diffusion noted. The substances used—mostly dyes—were introduced into the thimbles which were immersed in beakers of water and the amount of the substance which diffused at various times was recorded. We have obtained the following results with thimbles soaked in 80 %, 85 %, 90 %, 95 %, 100 % alcohol:

Sodium chloride had passed through all the thimbles in very considerable quantity after two hours.

Picric acid. After four days a certain amount of the substance had passed through the 80 % membrane. As in the case of the following substances the amount which diffused in that time increased with the strength of the alcohol employed in the treatment of the thimbles, the 100 % membrane showing the greatest permeability.

Potassium oxalate. None of the salt had passed through the 80 % membrane in four days; a certain amount diffused through the 85 % membrane.

Bismarck brown. None of the substance passed through the 80 % and the 85 % membranes; a certain amount diffused through the 90 % membrane.

Methylene blue. None of the substance passed through the 80 % and 85 % membranes; a trace diffused through the 90 % membrane.

Neutral red. None of the substance passed through the 80 %, 85 % and 90 % membranes; a certain amount of it diffused through the 95 % membrane.

Safranine behaved almost like neutral red.

Dextrin. None of the substance passed through the 80 %, 85 %, 90 % membranes; a trace diffused through the 95 % and a little more through the 100 % membranes.

Litmus. None of the substance passed through the 80 %, 85 %, 90 % and the 95 % membranes, some through the 100 % membrane.

Congo red did not pass through any of the above membranes in four days.

Autolysed yeast was used as the source for the antineuritic factor. The yeast after being washed free from wort was pressed out and placed in a large flask plugged with cotton wool in the hot room at 37° for a few days. The autolysed mass was filtered through a large Buchner funnel when sufficiently liquified and the brown filtrate thus obtained was dialysed for four days as described above and fed to rats which subsisted on a diet deficient in the antineuritic factor and which manifested signs of decline. Resumption of growth showed that the accessory factor had not totally diffused in that time. Failure to induce growth, on the other hand, showed that the active

substance had entirely passed through the membrane during the four days of dialysis.

As the source for the antiscorbutic factor lemon juice was employed which had previously been treated with an excess of calcium carbonate and filtered. This antiscorbutic solution, which was dialysed for three days, was tested on guinea pigs kept on a scorbutic diet of oats, bran and autoclaved milk. The administration of the doses was carried out about fourteen days after the animals had been put on the deficient diet, and in all the cases where the antiscorbutic factor diffused scurvy developed, in the other cases the onset of the disease was prevented.

A sample of the original material was always kept in a glass flask in the dialysing tank under the same conditions as the dialysing thimbles and was utilised for control purposes.

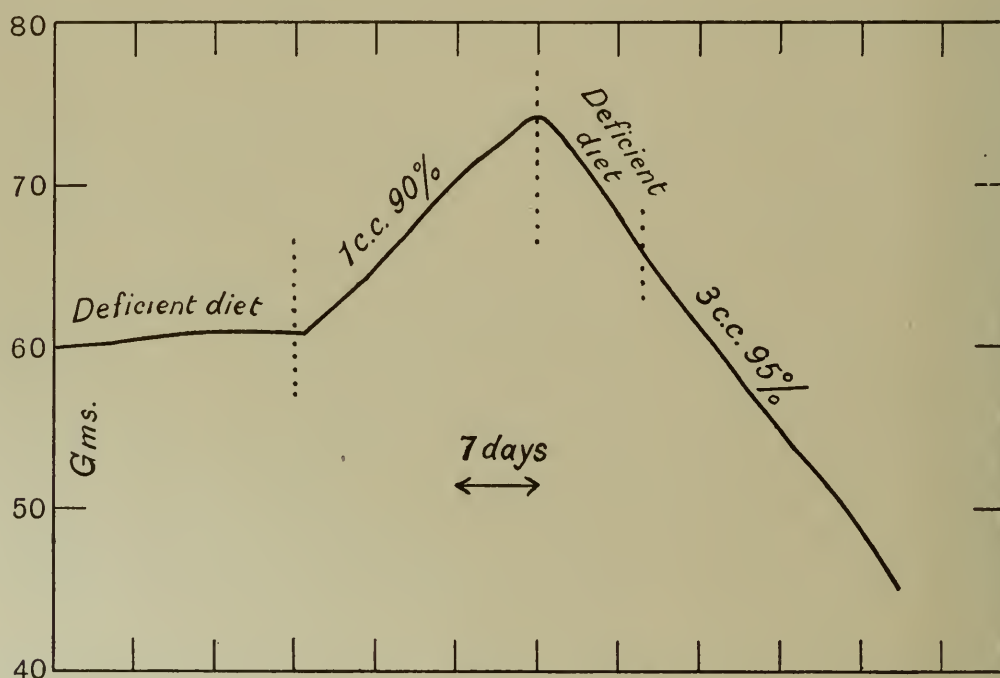


Fig. 1

Membranes of various permeabilities were tried until the one which allowed the free diffusion of the factors was obtained. In the case of both the antineuritic and the antiscorbutic factors it was found that the accessory factors would not pass through a 90 % membrane whilst they diffused entirely through a 95 % membrane in the time mentioned.

It will be seen from Fig. 1, which represents the growth curve of a rat kept on a diet deficient in the antineuritic factor, that an equivalent of 1 cc. of autolysed yeast dialysed through a 90 % membrane induced growth in the animal. As 1 cc. of autolysed yeast juice is approximately the minimum dose which is capable of inducing growth in a rat fed on a diet deficient in the antineuritic factor, one may conclude that very little of the factor diffused through this membrane in four days. The same animal was then placed again on the

deficient diet, and as was to be expected the rat declined in weight. The addition of 3 cc. of autolysed juice dialysed through a 95 % membrane for four days failed to promote growth, thus showing that the antineuritic factor had gone through entirely in this time. Other experiments have confirmed this observation: in all cases the factor failed to diffuse in any appreciable extent through a 90 % membrane but did so entirely through a 95 % membrane.

Similarly it will be seen from Fig. 2 that 7 cc. of decitrated lemon juice dialysed for three days through a 95 % membrane failed to prevent or even delay the onset of scurvy in a guinea-pig, whilst 3 cc. of the juice which had been dialysed through a 90 % thimble prevented the onset of the disease. The antiscorbutic factor therefore behaved much in the same way as the antineuritic factor. In this case also various experiments confirmed this result.

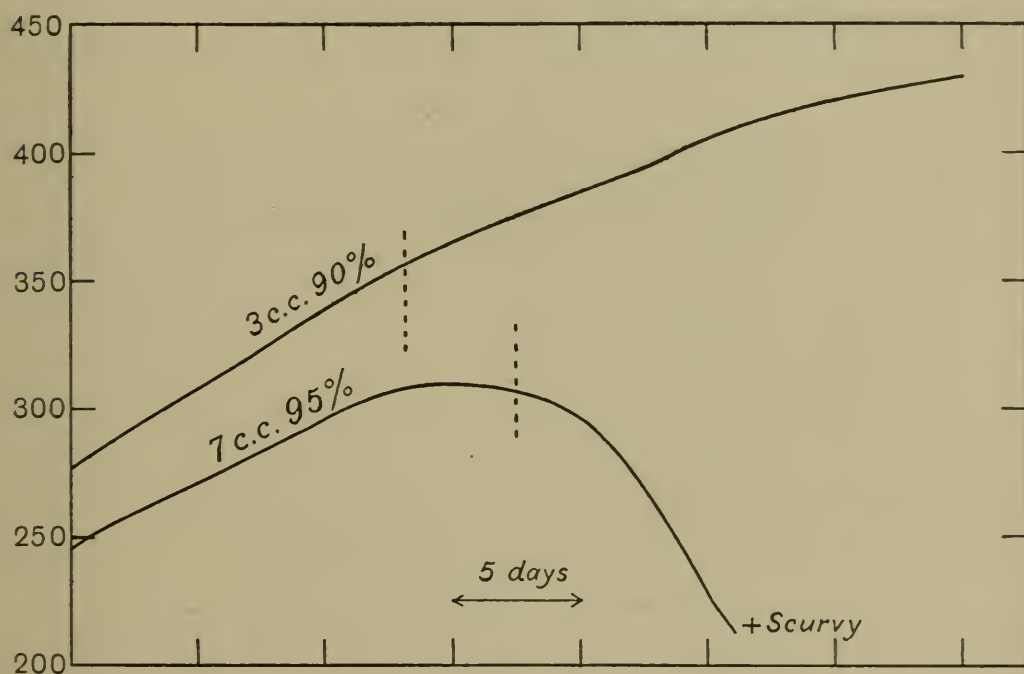


Fig. 2

A number of experiments with 35 %, 50 % and 70 % membranes showed that they were not permeable to the above accessory factors. These principles however passed freely through a 100 % thimble.

It is thus seen that the antineuritic factor in autolysed yeast juice and the antiscorbutic factor in decitrated lemon juice diffuse through membranes of such permeability as permit the passage of substances of such molecular dimensions as methylene blue, neutral red and safranine. From this one may conclude that the dimensions of the molecules of these factors or the molecules with which they may possibly be associated are of the order of that of a semi-colloid. There is, however, no evidence whether these molecules are simple or associated. It must also be pointed out that the above experiments were made without the application of pressure and only one source for each of the accessory factors was used. It would be of interest to investigate whether

factors such as hydrogen ion concentration, which influence the degree of dispersion, also influence the diffusibility of the antineuritic and antiscorbutic principles.

SUMMARY.

The antineuritic and the antiscorbutic accessory factors diffuse through a collodion membrane of such permeability as permits the passage of substances like methylene blue, neutral red and safranine. Membranes of lower permeability were found not to allow the diffusion of these factors. It is suggested that the active molecules whether simple or associated may be of a semi-colloid nature.

The expenses of this research were defrayed from a grant made by the Medical Research Council, to whom our thanks are due.

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Eijkman (1906). *Arch. Hyg.* **58**, 150.
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A NOTE ON THE RELATIVE ACTIVITY OF THE
FAT-SOLUBLE ACCESSORY FACTOR

IN COD-LIVER OIL AND BUTTER.

BY S. S. ZILVA, D.SC. LOND., PH.D. GIESSEN, F.I.C.,
AND
MASATARO MIURA, M.D. TOKYO.

(*From the Biochemical Department, Lister Institute.*)

THE therapeutic value of cod-liver oil, especially in the treatment of rickets, has long been appreciated by the clinician. The cause of this remarkable potency has not yet been satisfactorily explained, but the results of recent researches on the accessory food factors strongly favour the view that it is due to the presence of the fat-soluble factor in the oil. Professor Edward Mellanby's experiments with puppies point to the great probability that a deficiency of this factor in the diet is associated with the ætiology of rickets. Cod-liver oil, however, has shown itself always in the treatment of rickets to be superior to other substances which contain the fat-soluble factor. Results obtained by us in connexion with another inquiry seem to offer a possible explanation to this apparent inconsistency.

Our present knowledge of the distribution of the fat-soluble factor in nature is of a qualitative character. It is based on a technique which consists of feeding young rats on a diet adequate in every other respect, but lacking the factor. On such diet the animals decline, but on the addition of a substance containing the principle they resume normal growth. We have been engaged for some time in an investigation with the object of working out a method for the estimation of the fat-soluble factor on quantitative lines. From numerous experiments we have succeeded in ascertaining the necessary conditions under which results of a quantitative nature could be attained, and the method so far worked out, although not perfect, affords the

opportunity of comparing the relative content of the fat-soluble factor in various substances with a reasonable degree of accuracy. In this investigation we had the opportunity of testing a variety of substances, and we were struck by the extremely high potency of cod-liver oil. This was specially marked in the case of a sample of crude unrefined cod-liver oil, which was found to be 250 times as potent as butter. The samples of refined cod-liver oil which we examined, although not so active as the crude oil, were also far superior in their activity to butter. It is our opinion that this superiority in potency of cod-liver oil to other substances is responsible for the remarkable results achieved with it therapeutically. Unfortunately, in order to satisfy the requirements of the public there is a great tendency to produce brands of cod-liver oil which retain little of the characteristic taste and which appear almost colourless. In order to achieve this, very drastic means may often be employed which conduce to the partial or even total destruction of the accessory factor. In this connexion one may point out the great instability of the fat-soluble factor when exposed to air (Hopkins, 1920, Drummond and Coward, 1920) and ozone (Zilva, 1920), the latter substance being very often employed as a bleaching agent in many industries. It is hardly necessary to point out the serious consequences which might arise if in the process of refining this exceptional activity of the cod-liver oil were to be vitiated or destroyed in the manipulation of certain preparations.

The expenses of this research were defrayed from a grant made by the Medical Research Council, to whom our thanks are due.

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ORBITAL HÆMORRHAGE WITH PROPTOSIS IN EXPERIMENTAL SCURVY.

BY S. S. ZILVA, D.SC. LOND., PH.D. GIESSEN, F.I.C.,
AND

G. F. STILL, M.D. CANTAB., F.R.C.P. LOND.,
PHYSICIAN TO THE HOSPITAL FOR SICK CHILDREN, GREAT
ORMOND-STREET.

(From the Biochemical Department, Lister Institute.)

EXOPHTHALMOS or proptosis as a result of orbital hæmorrhage is an occasional but not frequent concomitant of scurvy in infants. Out of 64 cases¹ 6 showed orbital hæmorrhage, and in 5 of these there was proptosis; the proptosis in all cases was limited to the left eye. Of 11 cases recorded by various observers, 3 showed proptosis in both eyes, 3 in the right eye only, and 5 in the left eye only.

During experiments on monkeys by one of us (S. S. Z.) at the Lister Institute a similar occurrence was recently observed. On March 27th, 1919, the animal, a *Cercocebus fuliginosus*, was put on a scorbutic diet consisting of boiled rice, 250 g.; wheat germ, 50 g.; salts, 2 g.; milk (autoclaved one hour at 120° C.), half a pint daily, with the addition after July 7th of butter, 10 g. On May 21st there was reluctance to use the hind limbs. On the 27th the monkey remained in a lying position and would not sit up. Five c.cm. of unconcentrated decitrated lemon juice² were given and repeated the two following days. On the 29th there was some exophthalmos and hæmorrhage in the eyelids. By June 2nd under treatment with the decitrated lemon-juice the eye symptoms had almost disappeared and the legs were being used.

On omitting the lemon-juice the animal again showed hæmorrhage in the eyelids, but with further administration of the lemon-juice the monkey again became quite normal. On July 18th, after discontinuance of the lemon-juice for 29 days, the hind legs were again not used, and the next day purple discoloration of the gums

¹ Still: Common Disorders and Diseases of Childhood, 3rd ed., p. 114.

² THE LANCET, Jan. 4th, 1919, p. 17.

about the lower incisors appeared. There was also some diarrhoea. On the 21st hæmorrhage reappeared in the upper eyelids, and on the 22nd there was marked exophthalmos of the left eye, with such extreme eversion and bulging of the conjunctiva as completely to hide the cornea; the everted conjunctiva was covered with blood. The right eye showed no exophthalmos. The animal was lying on its side unable to sit up, but apparently not in pain. Decitrated lemon-juice of double strength was given in large doses (50 c.cm.) morning and evening, and next day there was much less exophthalmos, though still inability to sit up. On the 24th still larger doses of the decitrated lemon-juice were given, 60 c.cm. of the double strength in the morning and 50 c.cm. of quadruple strength in the evening. The same day the monkey began to move its legs and sat up, and the proptosis of the left eye was greatly diminished, and subsequently disappeared in a few days.

The recovery was extremely rapid; within six hours after the first administration of the double strength decitrated lemon-juice there was a marked improvement in the general aspect of the monkey, although the individual symptoms were still pronounced.

In this particular instance the exophthalmos affected the left eye, in agreement with the special tendency to left-side affection already observed in infants; but further observations will have to show whether this special liability of the left eye holds good in monkeys. Dr. A. Harden and Dr. S. S. Zilva³ have recorded an experiment on another monkey (*Macacus rhesus*) in which the scorbutic exophthalmos affected the right eye.

³ Journ. Path. and Bacteriol., vol. xxii., 1919.

The Lister Institute
of
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Collected Papers.

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3. **Details of the Technique adopted in following Weigl's plan of feeding lice infected with the virus of Typhus fever, by rectal injection.** By A. BACOT. *British Journal of Experimental Pathology*, Vol. III., 1922.
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6. **On the Pathology and Pathogenesis of *Spirochaetosis ictero-hæmorrhagica*.** By C. BASILE. *Journal of Pathology and Bacteriology*, Vol. XXIV., 1921.
7. **Blood Platelet Anti-Serum, its specificity and rôle in the Experimental Production of Purpura. Part I.** By S. P. BEDSON. *Journal of Pathology and Bacteriology*, Vol. XXIV., 1921.
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15. **The Toxigenic Features of Strains of the Diphtheria Bacillus isolated from Horses and from a Mule.** By G. F. PETRIE. *Journal of Hygiene*, Vol. XX., 1921-2.
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17. **Further Studies on the Production of Immunity in Rabbits against an Organism of High Virulence for the Species.** By J. PRATT-JOHNSON. *British Journal of Experimental Pathology*, Vol. II., 1921.
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19. **The Association of the Virus of Typhus Fever with the various blood elements.** By J. SÉGAL. *British Journal of Experimental Pathology*, Vol. III., 1922.
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*The late MR. ARTHUR W. BACOT,
Entomologist to the Lister Institute, London.*

N^o 1

IN MEMORIAM.

ARTHUR W. BACOT, F.E.S.,

Entomologist to the Lister Institute, London.

Born April 28th, 1866, Died April 12th, 1922.

By the death of Arthur Bacot, Entomologist to the Lister Institute, from typhus fever contracted in the course of experimental research into the ætiology of this disease in Cairo, medical entomology loses an investigator whose unique attainments had gained him world-wide repute. It was only in January of this year that Bacot and his colleague Arkwright proceeded to Cairo at the request of the Egyptian Government to continue researches into the nature and life-history of the typhus virus which in London were not fully satisfied by the employment of passage virus only. So far as technical methods went, the transference to Cairo meant to them simply a change of laboratory. They were accommodated at the Hygienic Institute in rooms placed at their disposal by the Director, Dr. Charles Todd, an old colleague of both. Precisely how Bacot, and shortly afterwards Arkwright, now happily convalescent, became infected, will perhaps never be known. In the course of their work they had found, in contrast to their own previous experience in London (see Atkin and Bacot, *Brit. J. Exper. Pathol.*, not yet published) and to that of Rocha-Lima, but in harmony with statements of Nicolle, that the excreta of typhus-infected lice were capable of conveying the disease to guinea-pigs, and it is just possible that infection arose from this source, especially as their work involved much handling of louse-boxes charged with virulent fæcal material. Both experts, however, knew well the risks they ran, and while providing against accidents so far as humanly possible, were not deterred from the pursuit of knowledge by the chance of some unlooked-for avenue of infection. To the long list of workers whose labours to solve the mystery of typhus fever have claimed the last penalty must now be added the name of Arthur Bacot.

At his death Bacot had all but completed his 56th year, and it was only 11 years ago that the opportunity came to him to bring his great knowledge of insect lore and bionomics—the fruit of a boyhood's hobby but the scientific passion of maturer years—to bear on the elucidation of vital problems of epidemiology. He was educated at the Birkbeck School, on leaving which he became a clerk in a city office, and there he remained till he was 45 years of

age. In his hours of leisure, however, he turned to his insect studies, and much of his early work in entomology was concerned with breeding experiments in Lepidoptera with special reference to analysis of Mendelian phenomena. Bacot was most successful as a breeder of insects for statistical analysis on Mendelian lines, and he was equally successful when at a later period in his career he undertook the study of blood-sucking insects in connection with human disease. At practically all times or at shortest notice Bacot could supply for his own experiments or those of others, lice, bugs or mosquitoes fed on his own person. Bacot rapidly became a lepidopterist of repute, and many of his contributions to entomology between the years 1890-1910 will be found in various entomological journals such as *The Entomologist's Record* and the *Proceedings of the Entomological Society of London*, of which he became a Fellow in 1901. In 1910 Bacot was requested by the Plague Research Commission to undertake a study of the bionomics of fleas, a subject on which the precise information available was but meagre. Further knowledge was indispensable to the elucidation of the rôle played by the rat flea in the transmission of plague from rat to man. Bacot undertook the work with enthusiasm, and by the provision of an assistant it was made possible for him to organise and supervise the multifarious experiments connected with the problem without totally severing his connection with the office stool. Within 18 months the ground had been largely covered, and the result was a compendium of knowledge on the life-history and bionomics of rat fleas which for all time must form the hunting ground of workers in this sphere. The experiments in connection with this work were carried out at Loughton, Essex, where Bacot lived, and the collated results are embodied in a lengthy monograph contributed to the *Journal of Hygiene*.

This research and its outcome so completely displayed Bacot's talents for undertaking and bringing to a conclusion a piece of systematic work under rigidly controlled conditions, that it became obvious that his attainments must be given scope for their fullest development by the opportunity of uninterrupted devotion to the medical aspects of entomology. He was consequently invited, in 1911, by the Governing Body of the Lister Institute, to accept the position of Entomologist to the Institute—a new post specially created for him. Henceforward he could devote himself solely to the development of his specialty and its application to current medical and epidemiological problems. To take an effective part in research requiring the services of variously trained experts, necessitates a very careful dovetailing of the various units concerned. To fit himself for such collaborative work Bacot was not long in familiarising himself with technical procedures hitherto new to him, such as sectioning and staining of insect tissues, and such was his handiness that he became most adept in this difficult art. A working knowledge of bacteriological technique also did not come amiss to him, so that in a remarkably short space of time he was able to undertake some complete problem unassisted or take an effective share in the problem of a team.

The exact mechanism of transmission of plague from rat to rat or rat to man by means of the rat flea was not clear.

The matter was clarified by a fascinating piece of work carried out by Bacot in conjunction with C. J. Martin. It was found that in the stomach of

the flea (*Xenopsylla cheopis* and *Ceratophyllus fasciatus*) which has sucked blood containing plague bacilli, the latter multiply rapidly and may form veritable masses of culture which fill up the proventriculus and even extend forward through the gullet. Fleas showing marked blocking of the proventriculus due to this cause are not prevented from sucking blood, as the pump is situated in the pharynx—they suffer indeed from thirst and bite with abnormal avidity—but the result of the pumping is that the already contaminated œsophagus is simply distended. At the conclusion of the pumping process some of the blood, carrying plague bacilli with it, is forced back into the wound. Success in transmitting plague experimentally to rats by plague-infected fleas was found to be largely, if not wholly, conditioned by a blocked proventriculus. Fleas in this condition are in danger of drying up if the temperature is high and the degree of saturation low, and it is probable that certain features of plague incidence in various parts of India, which appear to be correlated with local meteorological conditions, are in reality primarily dependent on the response of the blocked flea to such climatic variations.

Among other researches which occupied Bacot's attention at this time should be noted his work on the persistence of bacteria in pupæ and imagines of *Musca domestica* raised from larvæ which had been allowed to feed on a test bacillus. In the case of *Musca domestica* certain organisms, such as *B. pyocyaneus* supplied in the larval stage, were able to survive the cataclysm of the metamorphosis and appear in the pupæ and imagines. In the case of fleas, however, Bacot was unable to show that similar organisms could survive the pupal stage, and flea larvæ (*C. fasciatus*), taken from the bodies of mice dead from bubonic plague, and which had had opportunity to feed on faecal material contaminated with *B. pestis*, showed little evidence of the presence of this organism after dissection and staining of the stomach contents. The conditions in the larval interior of the flea do not appear to be favourable to multiplication of *B. pestis*, just as the larval interior of the fly does not appear to be favourable to the persistence of pathogenic bacterial species, such as *B. typhosus* (Ledingham, Tebbutt, etc.).

In July, 1914, Bacot proceeded to Sierra Leone to take part, as Entomologist, in an investigation into yellow fever, his services being placed at the disposal of the Colonial Office.

The outbreak of war interfered with the carrying out of the scheme of work proposed, but Bacot stayed in West Africa for a year studying the bionomics of *Stegomyia fasciata*, and later published a very complete monograph on the subject.

He returned to this country in October, 1915, and soon found himself immersed in studies on the bionomics of lice with a view to devising efficient methods of sterilising clothing and preventing louse-borne infection at the Front. Bacot's experiments on pediculicides were invariably carried out on himself under conditions similar to those met with in the field. He accepted the position of Honorary Adviser to the War Office on entomological questions, and henceforward he was constantly consulted on matters relating to insecticides.

Opportunity to bring his knowledge of lice and their habits to bear on a problem of great medical and military importance came in December, 1917,

when a Trench Fever Committee formed by the Director-General of the Army Medical Service was constituted under the Chairmanship of Sir David Bruce. Bacot had control of the entomological side of the inquiry, was responsible for the supply of infected lice, and superintended their feeding on trench fever patients and the collection of the louse excreta for further experiment.

In the course of this work Arkwright, Bacot and Duncan confirmed the observation of Töpfer that curious small bodies resembling *Rickettsia prowazeki* in typhus-infected lice were present in the gut lumen of lice fed on trench fever patients. These bodies did not appear in the louse till a period of eight or twelve days had elapsed after a feed on a trench fever patient, and it was only then that such lice were capable of again infecting man.

Trench fever disappeared with the war, and with it the opportunity of continuing research on the *Rickettsia* bodies associated with it. So many analogies, however, existed between trench fever and typhus fever, alike in connection with louse-transmission generally and association with *Rickettsia* bodies in particular, that Bacot and his colleagues resorted naturally to typhus research so far as this could be adequately prosecuted in London with passage virus. In 1918, and again in 1920, virus had been obtained from Ireland with which work was undertaken. In the latter year a further opportunity came to Bacot to study this disease when he joined, as Entomologist, the Commission appointed by the League of Red Cross Societies to prosecute typhus research in Poland under the guidance of Drs. Wolbach and Todd. While in Warsaw Bacot was alive to the possibility of finding evidence of the existence of trench or volhynian fever, and such evidence came in a surprising manner. While feeding on his own person lice collected from a public bath-house Bacot developed a sharp fever which necessitated his removal to hospital under suspicion of typhus fever.

During his stay in hospital he continued to nourish these lice and in due course they developed extracellular *Rickettsia* forms exactly similar to those met with in trench fever lice at home. For some months after recovery he apparently still harboured the trench fever virus in his blood and was able to infect otherwise clean lice by feeding them on himself. The proof that what he had suffered from in Warsaw was trench fever and not typhus was complete enough—a full account of his illness with experimental data appeared in the *British Medical Journal*—and his death from typhus fever almost exactly two years later is further proof, if proof be needed.

The work of the Warsaw expedition has recently been published in book form, and in it the claim of *Rickettsia prowazeki* to represent the actual virus of typhus fever is strongly urged. The chain of evidence, however, was not complete and it was therefore with lively anticipations that Bacot and Arkwright proceeded early in January of this year to Cairo to prosecute further researches into the ætiology of typhus.

Since his visit to Poland, Bacot had been able to practise the intrarectal infection of lice on the lines of Weigl's method, and had succeeded in keeping lice alive for fairly long periods on an intrarectal diet of human blood. By the aid of this new weapon Bacot and Ségal were able to watch the development of *Rickettsia* in lice thus infected, and to complete a chain of evidence by successfully infecting guinea-pigs with such lice, and thereafter demonstrating the

occurrence of *Rickettsia* in lice inoculated intrarectally with the blood of these infected guinea-pigs. These most recent experiments by Bacot in conjunction with Ségat and Atkin are in course of publication in this Journal.

No sketch of Bacot's life-work would be complete without some reference to his personal charm, his single-minded devotion to science, and his unfailing desire to be helpful to others. Colleague, acquaintance or casual visitor alike experienced at his hands that old-world courtesy now perhaps rarely seen among us. He leaves indeed a pleasant memory, and his works will live after him.

J. C. G. LEDINGHAM.

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1893-1910.

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- "Perils of Egg Life," 6, 1895.
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- "The Relationship of *Endromis versicolor* to the Sphingides," 7, 1896.
- "Notes on the Breeding of *Psilura monacha*," 7, 1896.
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- "Notes on the Life-history of *Papilio machaon*," 8, 1896.
- "Notes on the Early Stages of *Enodia hyperanthus*," 8, 1896.
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- "The British Liparid Moths," 10, 1898.
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- "Notes on the Larvæ of *Tephrosia bistortata* and *T. crepuscularia*," 10, 1898.
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- "Position of Egg laid by *Sphinx ligustri*," 10, 1898.
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- "Vitality of *Smerinthus ocellatus* bred in Confinement," 12, 1900.
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- "Larva of *Hyperchiria io*," 14, 1902.

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No 2

Stages of Rickettsia in the Sheep Ked : *Melophagus Ovinus*.

BY

DR. J. A. ARKWRIGHT AND MR. A. BACOT.

(Slides exhibited at a Laboratory Meeting of the Royal Society of Tropical
Medicine and Hygiene, Thursday, 17th November, 1921.)

Reprinted from the TRANSACTIONS OF THE ROYAL SOCIETY OF TROPICAL MEDICINE AND
HYGIENE, 1921. Nov. & Dec. Volume XV. Nos. 5 & 6. pp. 146-147.



At a Laboratory Meeting of the Royal Society of Tropical Medicine and Hygiene, held at the London School of Tropical Medicine, on Thursday, 17th November, 1921, at 8.15 p.m.,

Dr. J. A. ARKWRIGHT and Mr. A. BACOT exhibited slides showing stages of Rickettsia in the sheep ked—*Melophagus ovinus*.

Rickettsia melophagi was first described by NÖLLER in 1917. He stated that it was constantly present in the alimentary canal of *Melophagus ovinus* and was hereditary, being passed on to successive generations of the insect. The originally described and generally recognised form of this Rickettsia is a minute dumb-bell shaped organism about 0.4 micron in diameter, which is found in very large numbers lying close to the epithelial cells of the fore and mid gut, adhering to the cilia of the epithelium, and consequently arranged in rows at right angles to the surface of the lining membrane. The same part of the gut which is inhabited by the Rickettsia usually contains also great numbers of *Crithidia melophagia* which, however, lie nearer the centre of the lumen.

During the past year we have examined a number of *M. ovinus*, and in the case of the adult insect have been able to confirm the constant occurrence of enormous numbers of the typical form of *R. melophagi* in the alimentary canal. We have also seen other forms in very large numbers, which we regard as the same organism under different conditions or in different phases of its existence. The films and sections have been stained by Giemsa. The following varieties of form have been found, and specimens were shown :—

(1) The very small form seen in masses in the fore part of the gut may be rod-shaped instead of dumb-bell shaped, though still of approximately the same size.

(2) Much larger forms, which are more in evidence in places where there are few typical Rickettsia or flagellates. They are seen especially in smears of the hind gut of the adult insect and in the alimentary canal of the embryo. These relatively large bodies vary in size from about 1 to 2.5 microns, or even more, in diameter, are round or oval in shape, and are usually deeply stained at the poles or on one side, but unstained in the centre, though some are stained throughout. These forms were

at first regarded as an entirely different organism, but are in all probability *R. melophagi*.

(3) Intermediate forms between the minute and large forms are very numerous in some situations. These are short rods or oval forms, with poplar staining and clear centre. They vary very much in size and shape, and appear to provide abundant links connecting the extreme forms.

These links were first observed and are perhaps most often found in the gut of the embryo dissected out of the puparium and of the newly-emerged adult insect; in the latter, beside a very varied assortment of the large and intermediate forms, small groups of minute *Rickettsia* are often seen.

The variety of shapes and appearances seen has a striking likeness to that exhibited by some bacteria under different conditions, and this similarity strongly suggests the bacterial nature of *R. melophagi*.

We are indebted to Miss RHODES for the extremely accurate drawings for the accompanying plate.

[Reprinted from the TRANSACTIONS OF THE ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE, 1921, Nov. & Dec. Vol. XV. Nos. 5 & 6. Pp. 146, 147.]



Fig. 1.

Portion of smear of gut of an adult "ked"
after feeding.

× 1,500.

To face page 146.

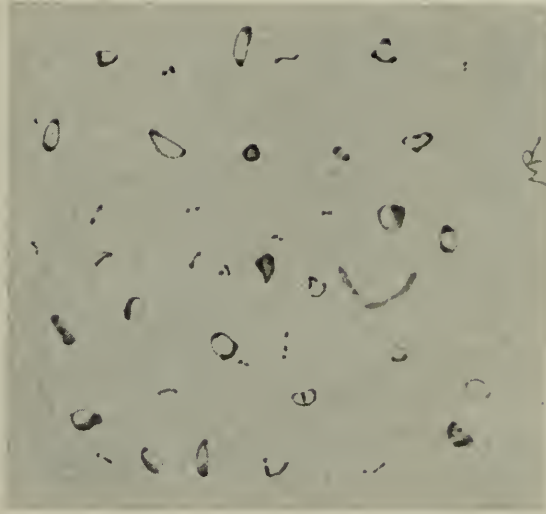


Fig. 2

Forms observed in smear of mid and hind
gut of newly emerged unfed "ked."

× 1,500.



Fig. 3

Forms observed in smear of mid gut of
newly emerged "ked."

× 1,500.



DETAILS OF THE TECHNIQUE ADOPTED IN FOLLOWING
WEIGL'S PLAN OF FEEDING LICE INFECTED
WITH THE VIRUS OF TYPHUS FEVER
BY RECTAL INJECTION.

A. W. BACOT, F.E.S.

From the Lister Institute, London.

Received for publication January 19th, 1922.

THE author had the advantage, when in Warsaw in the spring of 1920, of seeing Dr. Weigl demonstrate his plan of feeding lice by inserting a minute capillary pipette into the rectal opening of the insect and injecting a meal of blood. Dr. Weigl did not on this occasion demonstrate any attendant details of the sterilisation of the insect or the pipette prior to the operation, nor is any mention made of the necessity for such precautions in the summary in English of his paper (1920).

The alimentary tract of *Pediculus humanus*, and, so far as microscopic evidence goes, that of some species of lice occurring on animals other than man, is normally sterile; and experience shows that in the case of *Pediculus humanus* bacterial contamination of the gut is frequently, if not always, followed by the speedy death of the insect.

When breeding lice in gauze-covered boxes, evidence is obtained which suggests that bacterial contamination of the gut is one of the normal causes of death among captive lice, and that the death-rate from this cause is greatly accentuated by moist or very humid conditions, etc., in the boxes. It is therefore necessary, when feeding by rectal injection, to adopt measures to reduce the danger of infection, when inserting the pipette, to a minimum.

Sikora (1920) gives an account of the method she adopted in sterilising the lice she used for injection experiments (immersion in 1/1000 mercuric chloride followed by washing in iodine and hyposulphite of soda), but gives no indication as to whether the solutions were in diluted alcohol or water. It may be mentioned in this connection that lice can survive ten minutes' immersion in 85 per cent. alcohol. I have tried the method indicated by Sikora using 85 per cent. alcohol as the solvent, but personally have succeeded better by immersing the insects for from two to four minutes in 2 per cent. "lysol" (temperature about 60°–65° F.).

The lice* are transferred from the "lysol" to sterile water and removed thence to filter-paper placed in Petri dishes to recover. Activity is regained more rapidly by incubation at 90° F. for a few minutes.

* It was pointed out by Weigl that it is much easier to inject females than males; the death-rate from faulty technique speedily testified to the correctness of this warning.

To inject the infecting material or subsequent food (I have found whipped human blood most satisfactory), the louse is held in position under a slip of paper on a glass slide placed on the stage of a binocular dissecting microscope; the anal extremity of the insect should project a little way beyond the paper to allow of a clear view of the passage of the pipette. The magnification required is about sixteen diameters.

A finely drawn capillary pipette is redrawn in a minute flame and the tapering point cut off as nearly as possible at right angles by the pressure of a triangular pointed needle on a glass slide. One soon learns to judge the necessary external diameter (about 0.1 mm.) by comparison with the tapering point of the needle. The cut end of the tube must be smoothed by flaming, and this item of the technique I have found to require considerably longer practice than the correct insertion of the pipette into the rectal passage, which lies close beneath the dorsal skin. After loading, the pipette is inserted a short distance (a little beyond the last segmental incision) up the rectal passage and sufficient fluid is forced into the stomach by gradual pressure on a well-fitted rubber teat. Weigl used, and showed in his illustration, a small injection syringe, but in my experience a good teat is quite powerful enough.

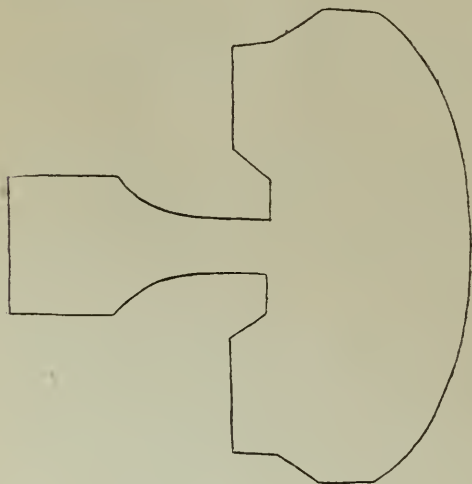


FIG. 1.

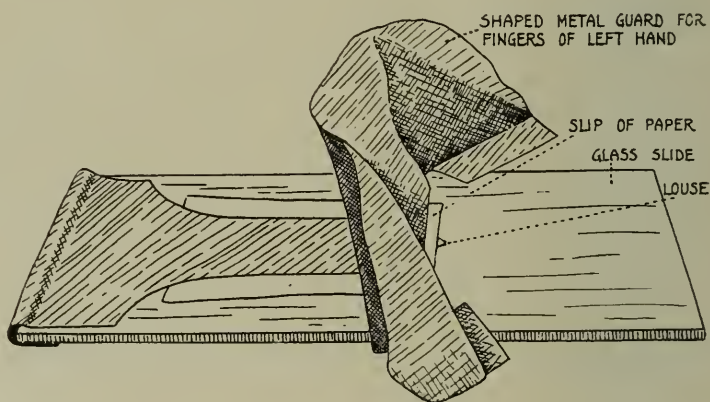


FIG. 2.

Until one has had considerable practice there is some danger, in working with infecting material, of pricking a finger before the pipette comes into the field of the microscope. A metal guard covering the fingers may be cut out of any thin soft metal (Fig. 1) and bent into shape as required (Fig. 2).

This safety device renders the operation somewhat slower, and is, of course, quite unnecessary when feeding with sterile blood.

The pipette is washed out after each injection with distilled water or salt solution, and then sterilised in boiling water before taking up the blood or infecting material for the next operation.

To Major Patton, I.M.S., I am indebted for the suggestion to touch the skin and hairs surrounding the rectal opening with a minute droplet of 85 per cent. alcohol immediately prior to the insertion of the pipette.

Two meals per day are necessary if the lice are to be incubated at 90° F.; one meal is sufficient at 80° F., and lice may be kept over the week-end for forty to fifty hours on a single meal if kept at 65° F. Lice have been successfully kept alive by this system of feeding for twenty-seven days, and are sufficiently well nourished to develop eggs and lay them if the temperature is high enough.

Healthy uninfected lice are generally easier to feed than those which are heavily infected with *Rickettsia prowazeki*. They further appear to be better nourished by their meals, and less liable to have the lower intestine obstructed by solid particles of excreta than the infected lice.

In view of the success which attends rectal feeding, the question, so frequently debated, of the use of salivary fluid is again raised. Presumably, owing to the extreme specialisation of the piercing and sucking apparatus, and the fact that the salivary ducts enter the base of the pocket beneath the palate, the salivary fluid does not obtain access to blood rectally injected. Its service, if any, as an aid to the digestion of the blood would seem, therefore, to be easily dispensed with, although, of course, its suggested use in connection with the prevention of coagulation is not called in question when the food given is defibrinated blood.

Lice which have been well fed rectally appear to have ravenous appetites when allowed to feed naturally, even on a monkey, but it is not perhaps safe to infer from this that rectal feeding does not assuage their appetites.

It was very noticeable during the course of experiments how short a time human lice survived when fed naturally on a *Macacus* monkey in comparison with those fed *per rectum* on whipped human blood.

A communication dealing with results of intra-rectal infection of lice with guinea-pig platelet emulsions containing typhus virus will appear in the next number of this Journal.

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110 4

WOOD TAR OILS FOR THE DESTRUCTION OF LICE (PEDICULUS HUMANUS) ON HAIR-CLAD AREAS.

By A. BACOT,

The Lister Institute of Preventive Medicine.

DURING the war, at the request of the War Office, I tested the efficiency of a by-product of the Department of Propellants, entitled "light wood tar oil," for use as an insecticide. It proved to be very serviceable for the destruction of lice, and I recommended it for use in the treatment of verminous heads as cheap, safe, and efficient.¹ It was also recommended as a basis for the preparation of culicifuges.²

In the spring of last year, while acting as entomologist to the Typhus Research Commission of the League of Red Cross Societies in Poland, I suggested the use of a sample of this oil, which I had with me in Warsaw, for use as an additional precaution in the clearance of lice from typhus patients. The Commission found it very effective, but were unable to obtain further supplies.

Since my return from Poland I have been unable to ascertain the existence of any further supplies of this particular oil, but through the courtesy of a late official of the Department of Propellants, I have been able to get into touch with two firms who are able to supply wood tar oils and wood oils of a somewhat similar description. These firms have kindly supplied me with samples with which I have carried out a number of tests, using a sample of the original "light wood tar oil" and an East Indian mineral lamp oil (called by the trade name of paraffin) as controls.

My object was limited to ascertaining by laboratory tests the probable efficiency of these oils for use in the destruction of lice on the hair of the body or the scalp.

Method of Testing.

1. A male, a female, and a nymph of *Pediculus humanus (capitis)*, the head louse, were immersed in the oils for two minutes and then placed on dry flannel to recover in the event

¹ Danger of Disease through Lice, London County Council Education Committee, May, 1919; and Lice, *School Hygiene*, March, 1919.

² The Comparative Efficiency of Certain Culicifuges, *Parasitology*, February 28th, 1919

of their survival. The result was that they were invariably killed.

2. As above, but immersed for thirty seconds only. The result was that all were killed.

3. Immersed as above for thirty seconds, but placed on absorbent filter paper after immersion. The males and females were killed, the nymphs sometimes recovered.

4. Instead of immersing the insects, a small drop, large enough to immerse a part but not the whole insect, was placed on the thorax of each specimen while it rested on absorbent filter paper. This experiment was performed twice. In both series the paraffin failed to kill any of the insects, which either never lost their activity or soon recovered it. The wood oils were considerably more effective, rendering most, if not all, the insects immobile for a time and generally killing one or more of the adults; the nymphs, which are more resistant, usually recovered.

There is therefore an element of chance in the killing of a louse by a drop which does not envelop the whole of the body, and this renders the thorough treatment of the hair down to the skin essential. Death depends on the penetration of the oil into the louse, and should it run off the wax-like surface of the skin or be rapidly absorbed by any fabric which comes in contact with the insect penetration is unlikely to ensue.

All the wood oils tested gave equally good results, but a watery fluid named "Furfurol" liquor, apparently consisting of water and some free oil, was found to be entirely ineffective in these trials.

The efficient oils are described as follows:

Messrs. Shirly Aldred and Co., Ltd., Worksop. Sample A, "wood creosote." Sample B, "light wood oil."

Messrs. R. W. Greeff and Co., Ltd., Thames House, Queen Street Place, London, E.C.4. Samples A, B, and C, Swedish wood tar oils.

As these oils are used for such trade purposes as flat-oiling wood to protect it from rot, etc., they should be obtainable at prices sufficiently moderate to enable them to be used more freely than the essential oils or proprietary articles which are frequently recommended. They should, in fact, prove even cheaper than the mixture of olive oil and paraffin which has been so widely recommended. If purchased in quantity, samples should first be obtained and tested to ascertain if any irritation of the skin is caused. The particular samples supplied caused my skin no irritation. It is necessary, however, to state that a very wide range of susceptibility exists in regard to the sensitiveness of the skin.

CONCLUSIONS.

My conclusions in respect of the use of such wood oils for the destruction of vermin in hair are as follows:

1. Nymphs should always be used in control tests, because, owing to the protection afforded by the double skin with which they are temporarily endowed when moulting, they are far more resistant than adult lice.

2. No absorbent material should come in contact with the treated area for a period (as long as possible up to an hour); greased or waxed paper or rubber-surfaced articles should for preference be used to cover the hair after treatment.

3. In treating hair-clad areas by spraying or other methods the oil must be used liberally so as to render the complete immersion of the vermin in droplets of the oil probable.

4. The oil should always be used neat, or if for any reason it is necessary to economize it, the fluid added should either be another oil or some fluid with which the oil is miscible. It must be borne in mind that volatile fluids, though frequently increasing penetration, shorten the period of action. Hence the effective nature of such remedies as olive oil and paraffin, where both the slow penetration of the heavy oil and the rapid evaporation of the light mineral oil are compensated for by mixing the two.

5. A repetition of treatment is always desirable within a few days. For typhus work a second treatment is essential in case eggs may have escaped the first treatment, because such eggs may hatch while the patient is still infective, and the lice after feeding be transferred to a nurse or doctor.

6. The objectionable odour of paraffin can always be covered by the addition of a small quantity of an essential oil or other scent such as oil of mirbane (nitro-benzol), camphor, etc. Camphor has an additional advantage in that it has the property of allaying skin irritation.



N^o 5

THE INFECTION OF LICE (*PEDICULUS HUMANUS*) WITH
RICKETTSIA PROWAZEKI BY THE INJECTION PER
RECTUM OF THE BLOOD PLATELETS OF
TYPHUS-INFECTED GUINEA-PIGS AND
THE RE-INFECTION OF OTHER
GUINEA-PIGS FROM THESE
LICE.

THE LATE A. BACOT, F.E.S., AND J. SÉGAL, M.D., L.Sc. PARIS, D.T.M. LOND.

From the Departments of Bacteriology and Entomology, Lister Institute, London.

Received for publication January 19th, 1922.

FOLLOWING Weigl's (1920) plan of feeding lice by rectal injections (Bacot, 1922), female specimens of *Pediculus humanus* were given an infecting meal of platelet material (Ségál, 1922) obtained by fractional centrifugation of the blood of typhus-infected guinea-pigs (G.P.). These lice were thereafter fed on defibrinated normal human blood by rectal injection, or for a portion of their infective period (in the case of Batches 32 and 33) naturally by allowing them to bite healthy *Macacus* monkeys. Although the insects fed greedily on the monkey they died within three to four days, apparently because of their inability to digest its blood.

The lice were incubated at 32° C. (90° F.) and fed twice daily for the major portion of the period following their infecting injection, but for some 40 or 56 hours in each week they were kept at room temperature (about 18° C., = 65° F.) and received only one meal during this period.

A number of the lice died showing a heavy bacterial contamination of the alimentary tract; in most *if not all* of the cases the growth of bacteria was almost certainly the actual cause of death.

From the fact that the cases of bacterial contamination were very capriciously distributed among the five batches of infected lice dealt with in this paper, and that the insects contaminated died within four to six days of the infecting meal; there seems no doubt that the source of the contamination was the platelet material* rather than the normal human blood on which they were fed subsequently to the infecting meal. This conclusion is supported by work now in progress.

The development of *Rickettsia prowazeki* was ascertained by dissecting out the guts of specimens which died and making smears of the teased-up guts, or by making smears of recently voided excreta, the former being the more certain of the two methods.

* Accidents which occurred during the preparation of the platelet material in certain cases render it possible that sterility was not absolute.

In a few instances *Rickettsia* bodies were present in the smears which showed bacterial contamination, but in most cases there had either been insufficient time for them to develop, or else they had been overwhelmed by the more rapidly growing bacteria.

Batch 31.

19 females of *Pediculus humanus* were injected with platelet material obtained from G.P. 72 (Chart 1).

5	specimens	died	on the	3rd	day.	All	negative.
5	"	"	"	4th	"	2 of which	showed a few thread or short rod forms of <i>R. prowazeki</i> .
3	"	"	"	5th	"	1 showed	doubtful but no definite <i>Rickettsia</i> bodies.
						1 showed	a few short rod forms (+).
						1 showed	a few thread forms (++).
2	"	"	"	6th	"	1	doubtfully infected.
						1	showed thread forms (+).

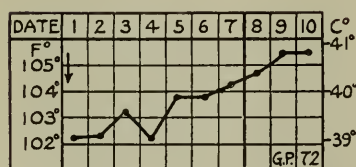


CHART 1.

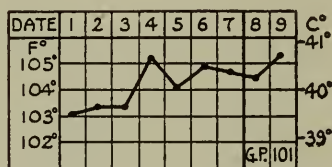


CHART 2.

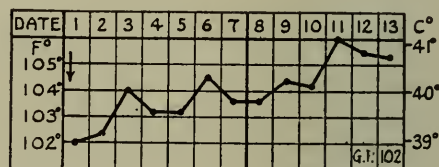


CHART 3.

1	specimen	killed	on the	9th	day.	Shown	short rod and thread forms (+ + + +).
						An emulsion	of this gut was injected into G.P. 101 (see Chart 2).
1	"	"	"	10th	"	Shown	short rod and thread forms (+ + + + +).
						An emulsion	of this gut was injected into G.P. 102 (see Chart 3).
						An emulsion	of its ovaries was injected into G.P. 103 (see Chart 4).
1	"	"	"	10th	"	Fixed	for sectioning (unexamined).
1	"	"	"	10th	"	Shown	short rod and thread forms (+ + + +).

Summary.—No contaminating bacteria. 6 out of 18 specimens examined showed *R. prowazeki*. Guinea-pigs 101, 102 and 103 were injected with emulsions from organs of the infected lice.

Results of injections of louse material into guinea-pigs.—G.P. 101 (Chart 2) injected intra-peritoneally with emulsion of gut. This G.P. was passaged by an injection of 2 c.c. of its blood into G.P. 110 and by an injection of an emulsion of $\frac{1}{3}$ of its brain into G.P. 111. G.P. 110 and 111 showed a positive reaction. G.P. 110 was tested for immunity within sixteen days of its infec-

tion by the injection of an emulsion of $\frac{1}{3}$ of the brain of an infected guinea-pig and gave a positive result. It is possible that this test was performed before immunity had been established, but it has been recorded by Da Rocha-Lima that guinea-pigs that have reacted to an infection of typhus virus have again responded to a second injection.

G.P. 102 (Chart 3) received an injection of an emulsion of the ovaries from the same louse the gut of which was used to infect G.P. 103. This G.P. was passaged by the injection of an emulsion of $\frac{1}{3}$ of its brain into G.P. 122 and 123, both of which showed a positive reaction.

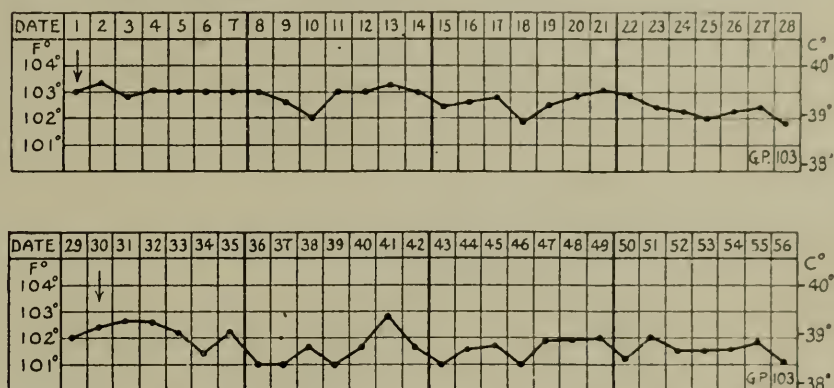


CHART 4.

G.P. 103 (Chart 4) received an injection of the gut of the same louse the ovaries of which were used to infect G.P. 102. G.P. 103 did not react to the injection, and was subsequently tested for immunity on the 30th day by an injection of brain emulsion from an infected G.P. As it failed to react to this we conclude that the animal was naturally immune.

Batch 32.

21 females of *P. humanus* were injected with platelet material from typhus-infected G.P. 84.

3 specimens died within 3 hours. Possibly owing to injury during manipulation.

3 „ „ 20 „ Cause not ascertained; probably injury or bacterial contamination of gut.

2 „ „ 3 days. 1 showed a few Rickettsia forms.
1 negative.

2 „ „ 4 „ The smear preparations were inadvertently destroyed.

5 „ „ 5 „ 3 showed bacterial contamination.
1 showed Rickettsia infection (+).
1 showed Rickettsia infection (++).

Smears made from the excreta of the 6 living specimens on the 7th day showed that some of the insects were heavily infected by *R. prowazeki*. These 6 lice were fed on a healthy *Macacus* monkey from this date until their death, the two last being killed only when they were too feeble to feed.

- 1 specimen died on the 9th day. Showed a heavy infection of small forms of *R. prowazeki* (++++).
- 1 „ (dying) was killed on the 10th day. Showed a heavy infection of threads and small forms of *R. prowazeki* (++++).
- An emulsion of the gut of this louse was injected into G.P. 121 (Chart 5).

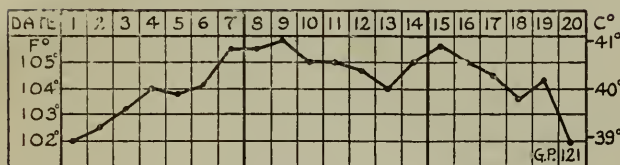


CHART 5.

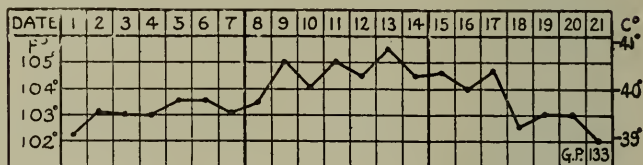


CHART 6.

- 1 specimen died on the 11th day. } Fixed for sections (unexamined).
- 1 „ „ „ 12th „ }
- 1 „ killed „ 13th „ Both heavily infected with *R. prowazeki*, mostly small forms (++++). Gut emulsions of one injected into G.P. 133 (Chart 6), and of the other into G.P. 134 (Chart 7).
- 1 „ „ „ 13th „

Summary.—9 died from unascertained cause, 3 died showing bacterial contamination, 9 died or were killed showing infection with *R. prowazeki*. Guinea-pigs 121, 133 and 134 were injected with emulsions of guts of these lice.

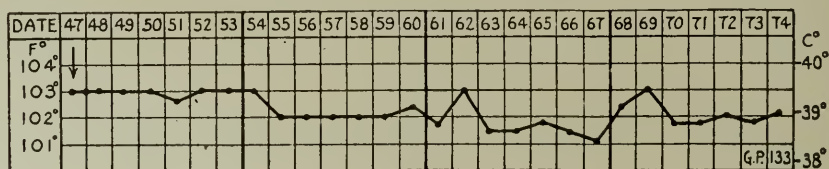


CHART 6A.

Results of injections of louse material into guinea-pigs.—G.P. 121 (Chart 5). Emulsion of gut intra-peritoneally. Died on the 20th day after injection.

G.P. 133 (Chart 6). Emulsion of gut intra-peritoneally. This G.P. was tested for immunity by the injection of $\frac{1}{3}$ brains on the 47th day after the first injection (Chart 6A).

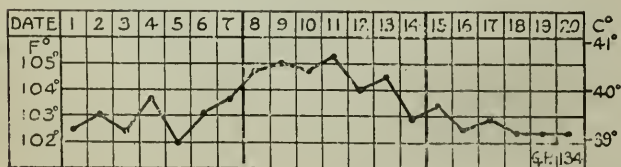


CHART 7.

G.P. 134. Emulsion of gut subcutaneously (Chart 7). This pig was tested for immunity by the injection of $\frac{1}{3}$ brain on the 35th day after the first injection, but the animal died on the 14th day without reaction from unascertained cause.

Batch 33.

12 females of *P. humanus* were injected with platelet material from typhus-infected G.P. 87. The preliminary wash in 2 per cent. lysol was omitted in the case of this batch. It is very doubtful, however, if the heavy incidence of bacterial contamination is attributable to this cause.

2 specimens died shortly after injection. From the second day after the infecting meal the remaining 10 were fed naturally by allowing them to suck blood from a healthy *Macacus rhesus* monkey. Within 5 days all had died, showing heavy bacterial contamination of their guts. In every case a few poorly stained *Rickettsia* forms were observed.

Batch 34.

18 females of *P. humanus* were injected with platelet material from typhus-infected Guinea-pigs 137 and 146.

3 specimens died within 2 days. Two were examined; both showed heavy bacterial contamination of the gut.

6 " " " 3 " All showed heavy bacterial contamination of the gut.

2 " " " 4 " Both showed heavy bacterial contamination of the gut.

2 " " " 5 " 1 showed heavy bacterial contamination of the gut.

1 showed a rather indefinite infection, probably of *R. prowazeki*.

1 specimen killed on the 7th day. Showed slight infection of *R. prowazeki* (+).

An emulsion of the gut was infected into G.P. 183 (Chart 8).

1 " died " 9th " Showing heavy bacterial contamination of gut and also poorly stained *R. prowazeki* (++) .

1 " " " 10th " Showing slight infection with *R. prowazeki* (+) .

1 " (dying) was killed on 14th day. Showing very heavy infection with *R. prowazeki* (++++) .

1 " was killed on 14th day. Was fixed for sectioning (not yet examined) .

Summary.—2 died unexamined; 11 died showing heavy bacterial contamination of gut; 1 died showing heavy bacterial contamination and also infection with *R. prowazeki*; 1 died showing possible infection of *R. prowazeki*; 3 showed definite infection of *R. prowazeki*.

Result of infecting G.P. 183 with emulsion of gut.—G.P. 183 was injected with an emulsion of the gut of a louse of Batch 34 (Chart 8). Material from this G.P. (183) was passaged (injection of an emulsion of $\frac{1}{3}$ of its brain) to Guinea-pigs 201 and 202 (Charts 9 and 10).

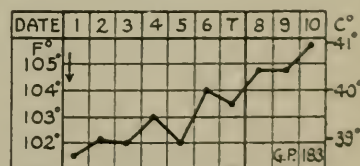


CHART 8.

G.P. 202 failed to react within 35 days and died before it could be tested for immunity.

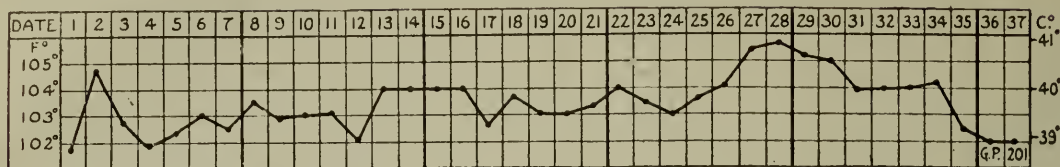


CHART 9.

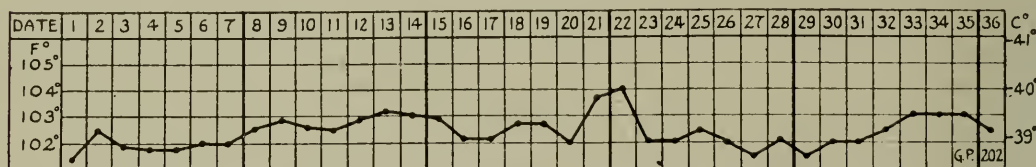


CHART 10.

Batch 35.

17 females of *P. humanus* were injected with platelet material from typhus-infected G.P. 141.

6 specimens died within 24 hours. } All showed a more or less heavy bacterial
1 specimen „ 36 „ } contamination of the gut.

1 „ killed on the 4th day. Showed an indefinite picture; very slight
if any development of *R. prowazeki*.

An emulsion of the gut was injected into
G.P. 182 (Chart 11).

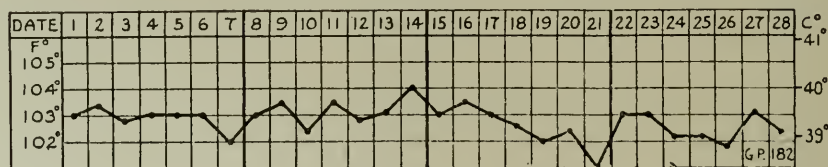


CHART 11.

1 specimen (dying) killed on 7th day. Showed (+ + +) infection of the gut-cells
with thread and bacillary forms of
R. prowazeki.

1 „ killed on the 8th „ Showed threads and small bacillary forms
(+ + + +) of *R. prowazeki*.

An emulsion of the gut was injected into
G.P. 191 (Chart 12).

1 „ „ „ 11th „ Showed minute bacillary forms of *R.*
prowazeki (+ + + +).

An emulsion of gut (+ + + +) was used
to infect another batch of lice, and an
emulsion of the ovary (+) was used to
infect G.P. 198 (Chart 13).

1 specimen (dying) killed on 12th day. Showed minute bacillary forms (++++)
of *R. prowazeki*.

An emulsion of the gut (++++) was
injected into G.P. 199 (Chart 14).

An emulsion of the ovary (+) into
G.P. 198.

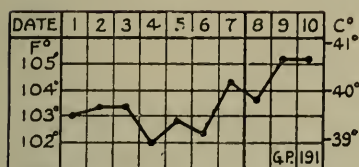


CHART 12.

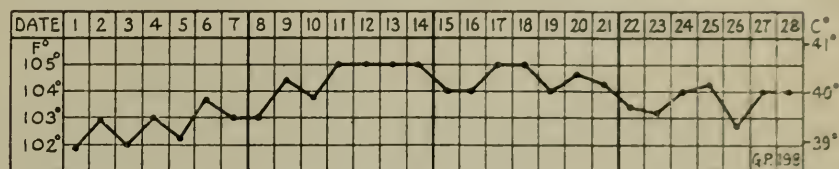


CHART 13.

1 specimen died on the 13th day. Showed minute bacillary forms of *R. prowazeki* (++++).

1 „ killed „ 13th „ Fixed for sectioning (unexamined).

2 specimens died „ 14th „ Showed small bacillary forms of *R. prowazeki* (++++) and few threads.

1 specimen killed „ 14th „ Showed small bacillary and short rod forms of *R. prowazeki* (++++).

Summary.—1 unexamined; 7 died showing bacterial contamination; 1 indefinite picture; 8 showed more or less heavy infection of *R. prowazeki*.

Guinea-pigs 182, 191 and 198 were injected with emulsions of organs of lice of Batch 35.

Results of infecting guinea-pigs and lice with emulsions of organs from Batch 35.—

G.P. 182. Infected with emulsion of gut (Chart 11) failed to react.

G.P. 191. Emulsion of gut (Chart 12).

G.P. 198. Emulsion of the ovaries of two lice injected on successive days (Chart 13).

G.P. 199. Emulsion of gut injected into testicle: died on the 12th day (Chart 14).

21 females of *Pediculus humanus* were injected with an emulsion of gut of a louse of Batch 35 and defibrinated human blood (Batch 36).

Smears of lice of this batch showed a more or less heavy infection of *R. prowazeki*. There were no cases of bacterial contamination.

Judging by the previous experience of one of the authors, the time occupied by the development of *R. prowazeki* in the louse when the platelets were used as the infecting material was only about $\frac{1}{2}$ to $\frac{2}{3}$ that required for an equivalent development when the lice were fed on an infected monkey or when whole blood of infected guinea-pigs was injected.

This is, no doubt, due to the greater concentration of the organism in the platelet material than in whole blood, and the time needed for lice to acquire a

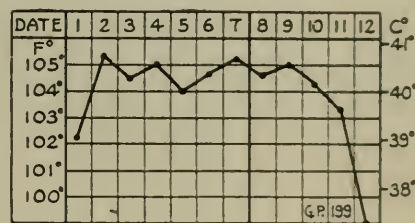


CHART 14.

heavy infection can be still further reduced by using as infecting material an emulsion of the gut of a heavily infected louse.

Apparently in whole blood the number of infecting units is so few that there are not enough gut-cells infected to enable them to be readily detected even at the end of their necessary incubation period. Later a secondary massive infection of the gut-cells takes place, probably as a result of the rupture of those first infected and the discharge of their contents into the lumen of the gut.

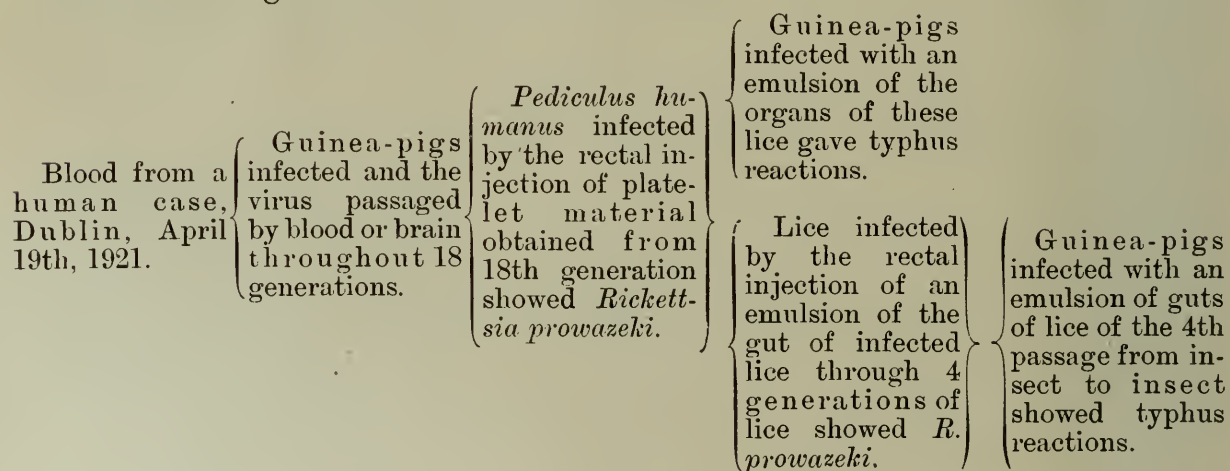
CONCLUSIONS.

(1) The injection of lice (*Pediculus humanus*) with a concentrated emulsion of platelets obtained by fractional centrifugalizations of the blood of a typhus-infected guinea-pig affords a sure and quick method of obtaining the development of *Rickettsia prowazeki* in these insects.

(2) The lice thus infected can be used to convey typhus fever to fresh guinea-pigs.

(3) The parallel development of typhus virus and *Rickettsia prowazeki* in successively passaged guinea-pigs is demonstrated and also in lice infected from guinea-pigs after 23 blood or brain passages.

The following succession has been obtained :



We desire to express our thanks to our colleagues Drs. Ledingham, Arkwright and Atkin for advice and assistance. Our grateful acknowledgments are also due to Dr. Atkin for supplying the virus, which he had already passaged through 8 generations of guinea-pigs before we started with the strain.

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ON THE PATHOLOGY AND PATHOGENESIS OF SPIROCHÆTOSIS ICTEROHÆMORRHAGICA.

By CARLO BASILE, D.T.M. (London).
Libero Docente—University of Rome.

From the Bacteriological Department of the Lister Institute, London.

INTRODUCTION.

THE study of the clinical symptoms of spirochætosis icterohæmorrhagica shows that this disease presents a different symptomatology in different cases. The impossibility of carrying out anatomo-pathological researches in all cases of human patients has led me to believe that the pathogenesis of the symptoms and the alterations in the tissues caused by this spirochæte may be rendered intelligible by a study of the disease experimentally produced in the guinea-pig, which is a susceptible animal.

The term *Spirochætosis icterohæmorrhagica* (Weil's disease) is based not only on historical grounds but also on the main clinical symptoms (jaundice and hæmorrhage) caused by this spirochæte when it manifests its full pathogenic action in a susceptible organism.

Material and Methods.

The virus of which I have made use in these researches was kindly placed at my disposal by Dr Ledingham, to whom I am extremely grateful. This virus was exceedingly virulent, guinea-pigs inoculated intraperitoneally dying after five or six days, though a few of them lived as long as twenty days after inoculation. The guinea-pigs were dissected immediately after death. Some were killed with chloroform, in order that the changes induced by the spirochæte might be followed approximately day by day. The organs were fixed in 10 per cent. formaldehyde, in alcohol, in Schaudinn's fluid, and in Zenker. A portion of each was treated by the original Levaditi method, or by the Volpino modification, or again by the Volpino-Bertarelli method.

Course of the Disease in Guinea-pigs.

Healthy guinea-pigs were inoculated intraperitoneally with the blood from the heart of infected guinea-pigs; the blood, as soon as it was drawn, was diluted in 5 c.cm. of a 2 per cent. citrate of soda solution previously sterilised; in some of the animals the inoculation was effected with a liver or lung emulsion.

The clinical side of spirochætosis icterohæmorrhagica in guinea-pigs

has been fully dealt with by previous writers, the course of the fever, the changes in the blood picture, the hæmorrhages, the albuminuria, and the jaundice being the principal symptoms. As previous authors have observed, the clinical picture is not always complete; jaundice may be absent in experimentally infected guinea-pigs. In my own work, which has been chiefly directed to the study of the pathology, I have almost constantly observed both in the serious and in the slight cases of experimental spirochætosis, the presence of hæmorrhages in the internal organs. In all my investigations this has been the most constant characteristic of the disease.

NAKED-EYE PATHOLOGY.

In acute cases the naked-eye pathology is almost always the same.

Jaundice shows itself in the skin of the regions without hair, and in its internal surface. The lymphatic glands of the groin and the axilla are hypertrophied. The hæmorrhages are the leading characteristic in all cases, acute or slight; those in the skin, the muscles, and the internal tissues may be very diffused; when localised they may be small, almost petechial, or they may be considerably larger. I have paid particular attention to the hæmorrhagic spots in the lungs, which are characteristic; Inada (1917⁷) and Kaneko and Okuda (1917¹⁰) have already described these lungs as having the appearance of the wings of a butterfly of the genus *Vanessa*. The hæmorrhage may attack the whole of a lobe of the lung. Sections show the hæmorrhagic spots to be situated round the bronchi and in the vicinity of the pleura. Hæmorrhagic spots are also seen in the serous membranes, in the stomach, in the intestine, in the region of the kidneys, and in the epididymis and vas deferens of the male guinea-pig. These hæmorrhagic spots seen with the naked eye show well-defined limits.

The liver to the naked eye showed nothing characteristic, and in the majority of cases its cut section was normal; in some guinea-pigs, however, I found hæmorrhagic spots.

The spleen in a few cases showed hypertrophy, but the cut section was of normal consistency.

The kidneys were in various stages of congestion, both of the cortex and the medulla; the suprarenal capsules were congested.

HISTOLOGY.

Lung.—Microscopically as well as to the naked eye the most constant and most characteristic lesions found in the guinea-pig were in the lungs. The cutaneous and subcutaneous hæmorrhages, the hæmorrhages in the serous membrane of the peritoneum, hæmorrhages in the stomach and intestines may be absent or unimportant, the changes in the liver and spleen may be slight if not negligible, but a constant feature was the hæmorrhages of the lungs and pleura.

I think that the hæmorrhagic state of the lungs, which may be very extensive,

is the most frequent cause of death of the infected guinea-pigs. The hæmorrhages in the lungs may occupy all the pulmonary alveoli; the smallest foci are formed of two or three pulmonary alveoli; the most serious hæmorrhagic foci are seen round the bronchi and may involve a large part of the pulmonary surface. All the blood-vessels are congested and full of red corpuscles.

The anatomical structure of the lung where there are no hæmorrhagic foci is usually well preserved. In preparations of the tissues fixed in formalin or sublimate, and stained with hæmatoxylin-eosin, or by the methods of Van Gieson, Hansen, and Twort, it is possible to recognise that some of the alveoli are distended and enlarged, and in these the alveolar wall is thin and may contain red corpuscles.

In the hæmorrhagic foci the quantity of blood is so great that it is not possible to distinguish the alveolar structure. In the foci, however, in which the process of reabsorption of the blood is going on, the structure is intact. In a guinea-pig which died seventeen days after inoculation I was able to distinguish the earlier hæmorrhagic foci from those which were formed subsequently. The first were found round the large bronchi and the blood was there in process of reabsorption, so that the soundness of the pulmonary tissue was readily distinguished. The more recent hæmorrhagic foci were in the neighbourhood of the pleura. Alveoli, even those at a distance from the hæmorrhagic foci may contain red corpuscles, and may also show internally a granular deposit suggesting œdema.

The cells of the walls of the alveoli show a well-stained nucleus, and no change is apparent in their protoplasm. Mononuclear cells containing phagocyted red corpuscles and polymorphonuclear leucocytes are occasionally seen.

In the epithelium of the blood capillaries mitotic figures may be detected. The epithelium of the bronchi and of the bronchioles shows proliferation. The bronchioles may also contain blood.

In some guinea-pigs I have also noted well-developed lymphatic nodules and lymphoid infiltrates mixed with red corpuscles.

These phenomena of lymphocytic reaction were more obvious round the bronchi and bronchioles and the pleura.

In all my preparations I have noted no conspicuous reaction of the connective tissue, which always appeared to me to be normal. The quantity of hæmosiderin was in relation to the state of degeneration of the red corpuscles. I have not observed fatty degeneration.

Spirochætes were very rare and there was no relation at all between the severity of the lesions and the number of spirochætes observed in lung sections.

Liver.—The presence of jaundice in spirochætosis icterohæmorrhagica has led students of this subject to turn their attention to the liver.

The observations hitherto published all agree in recognising that the changes in this organ are very varied. They differ in various patients, and are not in relation to the gravity of the jaundice. Garnier and Reilly (1917, 1918,²⁻⁷) have noted that serious jaundice may run its course without the discovery of important lesions of the hepatic cells, while in slight jaundice serious changes in these cells may be discovered.

My own observations in regard to the changes in the liver confirm those made by others. The liver is not an organ which invariably presents histological lesions in experimental spirochætosis icterohæmorrhagica. I group my observations in three series.

(a) In guinea-pigs which died from an acute form of the disease after five or six days, and in which the brain, lungs, and kidneys were highly congested, the liver appeared normal to the naked eye. Microscopically the congestion

was not remarkable. The chief finding was a lymphoid reaction around the blood-vessels. The hepatic cells showed minute vacuoles, and there were many cells with two nuclei; the necrotic foci were minute and surrounded by lymphoid cells. Fatty degeneration was not evident, and the connective tissue was normal.

(b) In other guinea-pigs which also died after five or six days from an acute experimental form of the disease, it was not possible to distinguish the lobules clearly. The cells partly retained their arrangement in columns. They were dissociated and were not swollen; the cellular plasma showed minute vacuoles. The nuclei of the liver cells were generally well preserved; some cells, however, showed two or three nuclei and other signs of degeneration with scattered granules of chromatin. The biliary capillaries were dilated, indicating bile stasis. The blood capillaries were full of red corpuscles and some polymorphonuclear cells. Phagocytosis of red cells and polymorphonuclear corpuscles by macrophages was very prominent; these were rounded and might be of large size; they may show a degenerating nucleus with scattered granules of chromatin.

Infiltration of lymphoid cells is also observable round the portal and arterial vessels but never in the central endolobular vessels. Again I have not noted fatty degeneration. Hæmosiderin was present in proportion to the quantity of red corpuscles and showed itself massed in small groups.

The endolobular connective tissue, in preparations well stained by Hansen's method, showed itself in the form of extremely fine fibres.

(c) In guinea-pigs which died sixteen days after inoculation, the liver, to the naked eye, was normal. Microscopically the arrangement in columns of the hepatic cells was not invariable; the hepatic cells were swollen, and their protoplasm showed vacuoles; the nuclei might be deeply stained or also swollen and showing signs of karyolysis. The necrotic foci were extensive and consisted of a residuum of cytoplasm in which there appeared remains of nuclei. The bile capillaries were dilated. The endolobular and perilobular connective tissue was very evident in the necrotic foci.

By the nitrate of silver method I was able to find in the liver spirochætes usually isolated and extracellular. Very rarely they were in cells which were in process of degeneration. I often found them also in small groups.

Kidneys.—The most interesting microscopic changes were observed in the convoluted tubules, the ascending loop of Henle, and in the glomeruli. These changes might indicate congestion or also necrosis. The cells which clothed the tubules were for the most part degenerated; in severe cases they were completely necrotic, and the tubules were delimited by the connective tissue which appeared to be sound, and which enabled the renal structure to be recognised. In some of the tubules the degeneration was so great that the lumen of the tubules was full of residua of nuclei, of red globules, and of leucocytes; these results which I have noted explain the presence of the characteristic cylinders which are found on examination of the urine in spirochætosis icterohæmorrhagica. I have observed also tubules containing hæmoglobin and red corpuscles. The blood vessels and the intertubular capillaries were congested. I have also found macrophages with included red corpuscles and leucocytes.

Mitosis in the uriniferous tubules did not appear to me to be very marked.

The changes in the glomeruli might be equally serious; the glomerular capillaries were much congested, the cells showed degeneration, their cytoplasm was vacuolar or granular, and their nuclear chromatin was reduced to granules. In the interior of the glomeruli also there were macrophages with red corpuscles and leucocytes. The endocapsular space showed itself in some glomeruli full of red corpuscles and leucocytes. The cells of the flattened Bowman's capsule were unchanged, although red corpuscles might be found in their midst.

In some kidneys I also noticed perivascular infiltration. I observed also hæmosiderin not only in the tubules but also in the glomeruli.

The interstitial connective tissue, stained by Hansen's method, was very plainly seen round the tubules and glomeruli.

In the different guinea-pigs which I examined I very rarely found spirochætes in the kidneys; when present they appeared to me to be chiefly localised in the lumina of the tubules.

Spleen.—Garnier and Reilly (1917-18²⁻⁷), and Martin and Pettit (1919¹¹), have already called attention to the function of the spleen in cases of icterohæmorrhagia; the spleen is the principal seat of hæmatophagic reaction.

Monti (1916¹³) was the first to recognise, on microscopic examination, that the lymphatic nodules of the spleen were frequently enlarged.

I have been able clearly to distinguish the enlargement of the lymphatic nodules. In the microscopic examination of the spleen I observed congestion, phagocytosis of red cells, and necrosis. When the nodules were surrounded by congested blood-vessels the phenomena of hæmatophagia were predominant; each cell might contain twenty or more phagocytosed red globules in various stages of degeneration. Around the lymphatic nodules were also to be found foci of necrosis formed of a granular substance containing red corpuscles and cellular residua.

The examination of preparations treated by the nitrate of silver method showed that the spirochætes are very rarely found in the spleen.

Suprarenal Capsules.—In my observations of these organs I have noted not only diffuse congestion but also a marked lymphocytic reaction.

Brain.—With the naked eye I have in all cases noted meningeal congestion. In fact at the microscopical examination the blood-vessels in the meninges as in the brain substance properly so called were deeply congested. The lymphocytic infiltration did not appear to me to be remarkable. Spirochætes were frequently to be found especially in the meninges.

Testicle and Epididymis.—The epididymis is the seat of a characteristic hæmorrhage; seen by the naked eye it is strongly hæmorrhagic, and, as has been described by Martin and Pettit (1919¹¹), the testicle would appear to possess a red cap.

On microscopical examination the tubular structure was found to be unaltered; the intertubular blood capillaries were deeply congested; in the intertubular spaces I have also noted a veritable collection of red corpuscles. The tubular epithelium was not much changed, and I have not observed any reaction in the connective tissue.

PATHOGENESIS OF THE SYMPTOMS.

The most important symptoms in man and in guinea-pigs are fever, hæmorrhage, and jaundice. The hæmatological data are not without interest, and, in fact, anæmia may be noted clinically in the second period.

The fever corresponds to the cycle of the spirochæte, or, more precisely, to the invasion of the organism.

Jaundice is not always present. Martin and Pettit classify the spirochætoses in this respect as icteric and non-icteric. Between these two forms there is a whole series of steps.

The hæmorrhages are less variable; sometimes there is simple congestion of the vessels, and this was found in all the guinea-pigs

experimentally infected, even in those which showed slight clinical symptoms. It is very probable they always occur also in man but that they are not always visible. Hæmoptysis is, in fact, well known.*

The jaundice and the hæmorrhages are the pathological data upon which is based all the symptomatology that is to be met with in spirochætosis icterohæmorrhagica. The hæmorrhages, which are at times exceptionally serious, do not appear to be determined by the direct action of the parasite; there is no intimate connection, in fact, between the violence of the hæmorrhage and the presence of spirochætes in the tissues.

In the lungs especially I have rarely been able to observe in the large hæmorrhagic foci well-preserved spirochætes, which are also very rarely present in the spleen and liver even when these organs are profoundly hæmorrhagic.

To explain this hæmorrhage, therefore, we must imagine some influence of a toxic nature on the endothelial cells of the blood capillaries. From the investigations carried out up to the present it does not appear that the spirochætes produce an exotoxin. Martin and Pettit believe that the hæmorrhages may be produced by an endotoxin contained in the body of the spirochæte, which is liberated by the destruction of the spirochæte following phagocytosis by the cells of the organism; in fact it is not infrequently found that these phagocytosed organisms break down and appear as granules enclosed in the cells. This phagocytosis would explain the disappearance of the spirochætes from the circulating blood and subsequently from the organs.

The degeneration and death of the spirochæte cause the liberation of toxic substances in the body of the cell, and owing to their action the cell is not long in showing phenomena of degeneration and necrosis. This condition which is repeated in the various organs leads us to think that it may be repeated also in the endothelial cells of the blood capillaries thus causing the hæmorrhagic state.

The phagocytic reaction, upon which I particularly insist, helps not a little to explain the jaundice.

Indeed the jaundice shows itself in the second stage of the disease; in experimental spirochætosis it is observed a few days before the animal dies. When the jaundice has appeared it is difficult to find spirochætes in the circulating blood—evidently they have been phagocytosed. Inada (1917⁹) has demonstrated that the infectivity of the blood in spirochætosis decreases gradually until it finally disappears

* In 1916, during my military service, I was able to follow the case of a soldier in hospital for jaundice. He had bloody sputum, very like the sputum in tuberculosis, suggesting a tuberculous infection in a jaundiced patient. For several successive days I made careful search for the tubercle bacillus, but without success; but later I found in preparations of the same sputum spirochætes morphologically identical with the spirochæta icterohæmorrhagica. This observation was described in a report on spirochætal jaundice presented to the Health Department of the Third Army Corps of the Italian Army in the field.

with the appearance of the jaundice. Garnier and Reilly (1917²) proved that three days after the appearance of the jaundice not only inoculations with peripheral blood but also inoculations with fluid from the spinal cord of spirochætosis patients gave negative results in guinea-pigs.

In the majority of cases the jaundice follows the hæmorrhage; the phagocytic reaction destroys the spirochætes whose poisons cause necrosis of the phagocytes, and hence also of the endothelial cells, thus causing congestion and hæmorrhage.

The red corpuscles are disintegrated and destroyed by phagocytes and by the action of the toxic substance set free by spirochætes.

The greater part of the hæmoglobin is transformed into bilirubin, and I think that this transformation can take place not only in the liver but even in the blood-vessels themselves and in other organs. I do not know how otherwise to explain why in severe cases of jaundice the bile capillaries of the liver are not dilated and no other serious lesion is found in this organ, while in slight cases of the disease we have serious histopathological manifestations in the liver. That the hæmoglobin can be transformed into bile pigment not only in the liver but also in the peripheral circulation has been demonstrated by the experiments of Hooper and Whipple (1916¹⁴), who have proved that hæmoglobin can be rapidly changed into bile pigment in the local circulation of the head, of the neck, of the thorax, and of the pleural and peritoneal cavities without the participation of the liver. These writers also consider it very probable that the endothelium and also the mesothelium (serous cavities) may be able to transform hæmoglobin into bile pigment; they also advance the hypothesis that this property may also exist in cellular protoplasm in general.

We may therefore hold the opinion that in spirochætosis ictero-hæmorrhagica the hæmoglobin, produced by the marked phagocytic action and by the action of the toxic substance set free by spirochætes above described, may be converted into bile pigment without the assistance of the liver.

Garnier and Reilly (1917⁵) (who were the first to observe the contrast between severe jaundice with the liver histologically almost normal, and slight jaundice with serious histopathologic manifestations in the liver, being supported by the fact recognised by themselves that bile *in vitro* has a lytic action on the spirochætes) have considered that the bile stasis, by injuring the vitality of the spirochætes, hinders their pathogenic action on the hepatic cells, and therefore that the slight change or the absence of change in the hepatic cells in severe cases of jaundice is in relation to the bile stasis, while in those cases in which the bile stasis is absent, the spirochætes can develop all their pathogenic action on the hepatic cells. But if this fact explains the action of the bile on the spirochætes it does not explain the cause of the jaundice. The jaundice in spirochætosis icterohæmorrhagica may

even have a mechanical cause, namely, inflammation and swelling of the duodenum and the papilla of Vater.

Beside the hæmatophagic reaction we also find the lymphocytic reaction. This occurs not only in the spleen and in the principal lymphatic glands but also in all the lymphatic nodules of the organs, as in the lungs, in the liver, in the kidneys, etc. The lymphoid reaction joined with the phagocytic reaction contributes to the destruction of the spirochætes and to the defence of the organism. These two reactions together with the relative absence of nucleated red globules in the spleen and in the peripheral blood explain to us the anæmia which so frequently presents itself in the secondary period of spirochætosis icterohæmorrhagica, and it also explains the leucocytosis which is so constantly revealed at the hæmatological examination.

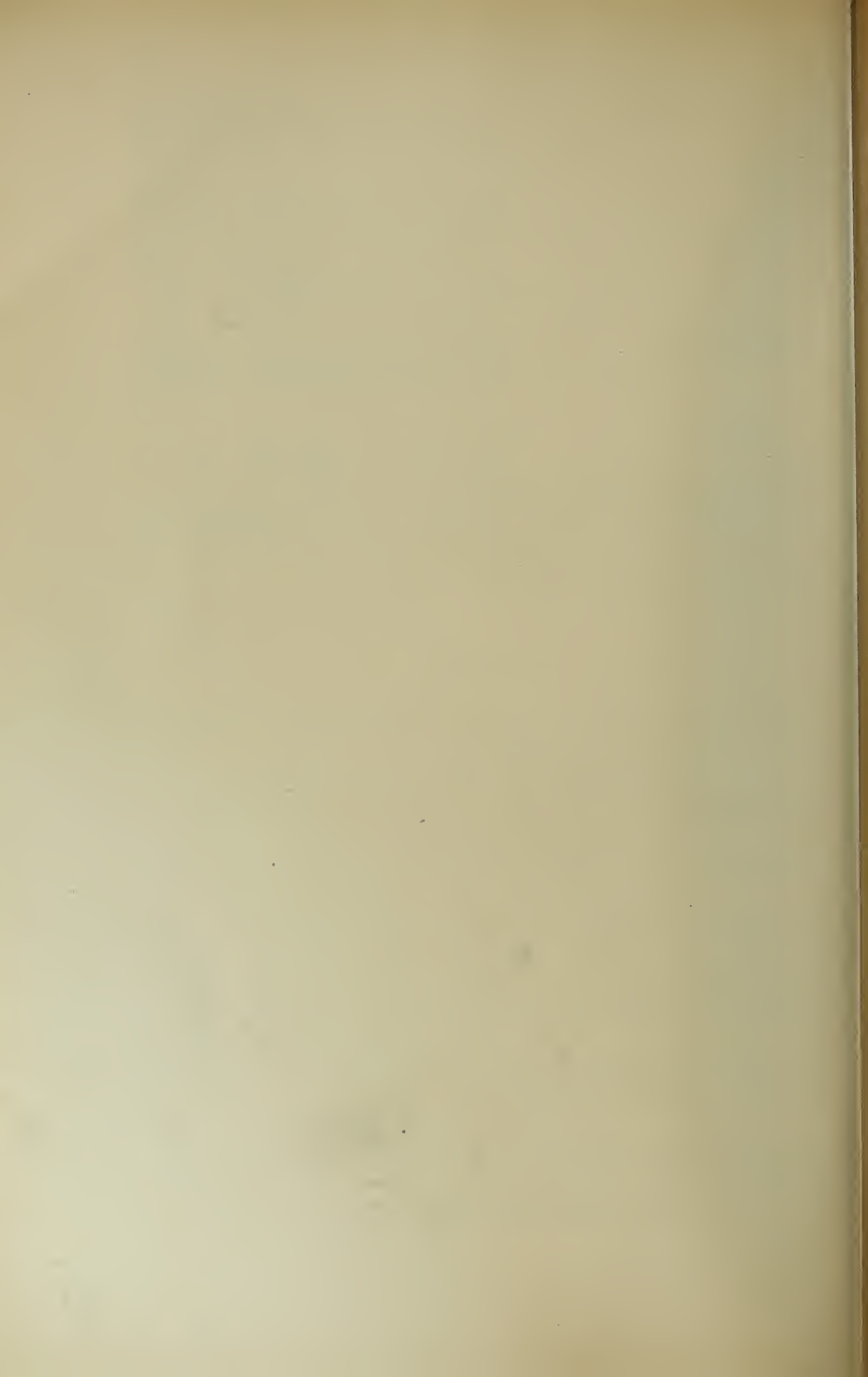
Addendum.

Since this paper was completed I have seen the recent communication by M'Nee (1920¹²) on the morbid anatomy and mechanism of production of the icterus in spirochætal jaundice in man. I appreciate his conclusions so far as the human infection is concerned, but believe that the hæmorrhages which in infected guinea-pigs are far more abundant and striking than in man, play a very important part in the pathogenesis of the icterus.

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No 7

BLOOD PLATELET ANTI-SERUM, ITS SPECIFICITY AND ROLE IN THE EXPERIMENTAL PRODUCTION OF PURPURA.*

By S. PHILLIPS BEDSON, M.D., M.Sc. (Dunelm).

Assistant Bacteriologist, Lister Institute, London.

Part I.

ALTHOUGH anti-platelet sera were first prepared as far back as 1905 (Marino, 1905¹), and have since been investigated by numerous workers, our knowledge of them is still far from complete.

The earlier investigators in this field, Chevrel and Roger (1907²), Le Sourd and Pagniez (1908³), Sacerdotti (1908⁴), Cole (1908⁵), Stschastnyi (1909⁶), have shown that these sera are definitely species-specific, but the question of their cell-specificity was left by them a matter of doubt.

It is true that Sacerdotti claimed to have demonstrated the presence of a specific platelet antibody in anti-platelet sera by means of absorption experiments, but the findings of Stschastnyi did not support this. Furthermore, Aynaud (1911⁷), repeating the work of Sacerdotti, but on a more extensive scale, was unable to confirm his findings and came to the following conclusions:—

1. Anti-platelet sera are species-specific.
2. *In vitro* they are capable of agglutinating platelets, and with the aid of complement, of lysing them to a certain extent. Similarly they are agglutinating and lytic for red cells.
3. Anti-red-cell sera are similarly endowed, agglutinating and lysing platelets as well as red cells.
4. It is impossible to demonstrate the presence of a specific platelet antibody in anti-platelet sera by means of absorption.
5. The specificity, *i.e.*, the cell specificity, of anti-platelet sera *in vivo* is purely relative.

Early in 1914 it was shown by Ledingham⁽⁸⁾ that an anti-guinea-pig-platelet serum was toxic for the guinea-pig, giving rise in this animal to a condition closely resembling purpura hæmorrhagica in man. Following up this observation it was shown (Ledingham and Bedson (1915⁹)), that, although toxic for the animal, sera prepared against other blood elements, *e.g.*, anti-red-cell and anti-white-cell (polymorphonuclear mainly), were unable to produce this striking pathological picture, and that here again the species specificity of anti-platelet sera was quite definite. The characteristic feature of the blood picture in these purpuric animals was the early and extensive fall in the number of platelets, other sera producing little

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or no change in this respect. This work has been repeated and confirmed both in America by Musser and Krumbhaar (1916¹⁰), Lee and Robertson (1916¹¹), Gottlieb (1919¹²), and in Japan by Watabiki (1917 and 1918¹³), but no new facts have been brought to light by these investigations. Now here we have demonstrated beyond doubt a specific action on the part of anti-platelet sera—the production of purpura in the animal—yet when we come to investigate such a serum *in vitro* it is found to agglutinate and lyse red cells at a higher titer than that at which it agglutinates and alters platelets. This hæmagglutination takes place also in the animal, a “clumped” appearance of the blood being a constant and striking feature in experimental purpura, so much so that this hæmagglutination was thought to play a prominent part, in a purely mechanical way, in the production of the hæmorrhage. Is there then a specific antibody for platelets in anti-platelet sera, and if so what part does it play in the production of purpura? It was in the hope of solving these questions that the following work was undertaken.

SCHEME OF WORK.

It was decided to prepare anti-sera to the various blood elements of the guinea-pig and to titrate these sera, by means of the agglutination reaction, against the various antigens used in their preparation. Having defined in this manner the inter-relationship of these sera, it was proposed to proceed to absorption experiments, particularly with anti-platelet sera, and finally to work out the effect of these various sera, absorbed and unabsorbed, in the animal. I shall deal with the serological portion only of the investigation in this communication.

PREPARATION OF ANTI-SERA.

The greatest care was taken to have the antigens in as pure a state as possible. In the case of the platelets, this was obtained by repeated fractional centrifugalisation of the citrated blood and the suspension was not considered satisfactory until microscopically free from red cells. The red cells were obtained from defibrinated blood. The leucocytes (polymorph) were obtained by inoculating guinea-pigs intra-peritoneally with sterile broth, killing them six to seven hours after and washing out the peritoneal cavity with 1 per cent. sodium citrate in saline, care being taken to avoid bleeding from cut vessels into the peritoneal cavity. This was probably the least satisfactory of all the antigens employed, as it proved exceedingly difficult to obtain a suspension of leucocytes entirely free from red cells on microscopical examination. These sera were produced in rabbits in the usual manner and were heated to 56° C. for thirty minutes and filtered through Berkefeld candles before storing in ampoules. In all five sera were prepared—anti-platelet, anti-red-

cell, anti-leucocyte (polymorph), anti-serum (precipitating), and anti-whole-blood (citrated blood).

TITRATION OF SERA AGAINST THE DIFFERENT ANTIGENS.

Technique.—In these agglutination experiments the sera had been heated to 56° C. for thirty minutes and filtered through a Berkefeld candle beforehand. A series of dilutions of the serum was made in small agglutination tubes, each tube containing 1·0 c.c. of serum dilution. To each tube was then added 1·0 c.c. of the antigen suspension in question, the tubes shaken and placed in the incubator at 37° C. for two hours. The first reading was then taken and the tubes allowed to stand at room temperature overnight when the second reading was made. In the preparation of the suspension of the different cells for these titrations, the same care was taken as in the preparation of the antigens for inoculation. The platelet and the leucocyte suspensions were standardised by means of opacity, whereas in the case of the red cells a 2 per cent. suspension in normal saline was employed. Owing to the rapidity with which leucocytes and red cells settle out some difficulty was experienced in reading the results. However by observing the deposit and then shaking up the contents of the tubes and comparing with the controls, the degree of agglutination can be accurately gauged. Some trouble was experienced at first in obtaining a satisfactory leucocyte suspension, spontaneous clumping occurring quite frequently. If, however, care is taken to dilute the peritoneal exudate of the guinea-pig with a large excess of citrate solution in the first instance, this difficulty is not encountered. In the Tables I. to V. the degree of agglutination has been recorded as follows :—

- ++++ = Complete agglutination and sedimentation.
- +++ = Almost complete agglutination. Supernatant fluid not quite clear.
- ++ = Agglutination with commencing sedimentation.
- +
- = Well-marked agglutination. No sedimentation.
- ± = Strong trace of agglutination.
- ⊕ = Faint trace of agglutination.

1. Against Platelets.

TABLE I.

Serum.	Final Dilution of Serum in each Tube.									Control. (Saline + suspension.)
	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	1/2560.	
Anti-platelet	+++ ++++	+++ ++++	++ +++	+ ++	± ++	— +	— ⊕	— —	— —	— —
Anti-red	± ++	± ++	⊕ +	— ±	— ±	— ⊕	— —	— —	— —	— —
Anti-leucocyte	— —	— —	— —	— —	— —	— —	— —	— —	— —	— —
Anti-serum	++ +++	+ ++	± +	⊕ ±	— ⊕	— —	— —	— —	— —	— —
Anti-whole blood	+++ ++++	+++ ++++	++ +++	+ ++	± +	?⊕ ±	— ⊕	— —	— —	— —
Normal rabbit	— ⊕	— —	— —	— —						

The outstanding feature brought out in Table I. is the inability of the anti-leucocyte serum to agglutinate platelets. All the other sera agglutinate to a greater or less extent, the highest titers being those of the anti-platelet and the anti-whole-blood sera. The agglutination of platelets by an anti-red-cell serum has been observed by many independent workers, and both Sacerdotti (1908⁴) and Aynaud (1911⁷) stated that a precipitating serum (anti-serum) was capable of clumping platelets in low dilutions. This may possibly be due to the fact that serum contains some platelet antigen liberated during the process of coagulation. This is borne out by the observation of Aynaud, that an anti-plasma serum is unable to fix complement with platelets as antigen.

2. Against Red Cells.

TABLE II.

Serum.	Final Dilution of Serum in each Tube.									Control (Saline- suspension.)
	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	1/2560.	
Anti-platelet	++++ ++++	++++ ++++	++++ ++++	+++ ++++	++ +++	+ ++	± +	- -	- -	- -
Anti-red-cell	++++ ++++	++++ ++++	++++ ++++	++++ ++++	++++ ++++	++++ ++++	+++ ++++	+ ++	± +	- -
Anti-leucocyte	++++ ++++	++++ ++++	++++ ++++	+++ ++++	++ +++	± +	- ±	- -	- -	- -
Anti-serum	++++ ++++	++++ ++++	++++ ++++	+++ ++++	+ ++	± +	± ±	- -	- -	- -
Anti-whole blood	++++ ++++	++++ ++++	++++ ++++	++++ ++++	++++ ++++	+++ ++++	+ ++	± ++	± ±	- -
Normal rabbit	- -	- -	- -	- -						

It is seen from Table II. that all five sera agglutinate red cells to a considerable extent, the highest titers recorded being those of the anti-whole-blood and anti-red-cell sera, which is what one would expect. It is difficult to explain the agglutination of red cells by a precipitating serum (anti-serum).

3. Against Leucocytes.

In Table III. the reverse of what was noted in Table I. is seen, namely, the inability of an anti-platelet serum to agglutinate leucocytes. The anti-serum (precipitating) serum is almost without effect and the highest titer is given by the anti-leucocyte serum.

TABLE III.

[illegible]

4. Against Serum.

When varying dilutions of guinea-pig serum are put up with constant quantities of the anti-sera (0.02 c.c.) it is found that the precipitating serum produces a heavy precipitate even at comparatively high dilutions, whereas the other four sera have comparatively little effect, with the exception of the anti-whole blood serum, which produces a definite cloudiness with little or no precipitation even with high dilutions of the antigen. These results are given in Table IV.

TABLE IV.

Serum.	Dilutions of Antigen.									Control (Saline).
	1/80.	1/160.	1/320.	1/640.	1/1280.	1/2560.	1/5120.	1/10,240.	1/20,480.	
Anti-platelet	+ ++	± +	?+ ±	- +	- -	- -	- -	- -	- -	- -
Anti-red cell	- +	- ±	- ±	- +	- -	- -	- -	- -	- -	- -
Anti-leucocyte	- ±	- +	- -	- -	- -	- -	- -	- -	- -	- -
Anti-serum	+++ ++++	++ ++++	+++ ++++	++++ ++++	++++ ++++	++ ++++	++ ++++	± ++	?+ +	- -
Anti-whole blood	± ++	+ +	- +	- ±	- ±	- ±	- +	- +	- -	- -
Normal rabbit	- -	- -	- -	- -						

An interesting feature of the above recorded results is the inability of an anti-platelet serum to agglutinate leucocytes, and the fact that the converse of this is also true. Considering, then, these two antigens alone—platelets and leucocytes—the anti-platelet serum is seen to be specific, but when we consider the third antigen, red cells, this specificity is no longer apparent. Anti-platelet serum agglutinates red cells as well as platelets, and vice versa. It was decided therefore to see whether a specific antibody could be demonstrated in this serum by means of absorption. The following experiment was carried out:

Tube 1. 0.5 c.c. Anti-platelet serum + 2.0 c.c. 50 per cent. suspension of guinea-pig red cells.

Tube 2. 0.5 c.c. „ „ + 2.0 c.c. suspension of guinea-pig platelets (from six guinea-pigs).

Tube 3. 0.5 c.c. „ „ + 2.0 c.c. saline (control).

These three tubes were incubated at 37° C. for two hours, centrifuged, and then titrated against the two antigens with the following result:—

TABLE V.

<i>Anti-platelet serum unabsorbed.</i>										
Antigen.	Final dilution of serum in each tube.									Control. (Saline + suspensions.)
	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	1/2560.	
Platelets {	+++ ++++	+++ ++++	++ ++++	++ ++++	+ +++	?+ ++	- ±	- -	- -	- -
Red cells {	++++ ++++	++++ ++++	+++ ++++	+++ ++++	++ +++	++ ++	+ ++	?+ ±	- -	- -
<i>Anti-platelet serum absorbed with red cells.</i>										
Platelets {	+ ++	++ ++++	++ ++++	+ ++++	+ ++++	+ ++	- ±	- -	- -	- -
Red cells {		- -	- -	- -	- -	- -	- -	- -	- -	- -
<i>Anti-platelet serum absorbed with platelets.</i>										
Platelets {	+ ++	+ +	- ±	- -	- -	- -	- -	- -	- -	- -
Red cells {	++++ ++++	+++ ++++	++ +++	+ +++	± ++	- ±	- -	- -	- -	- -

It will be seen that absorption with red cells has removed all the red cell agglutinins but has left untouched the platelet agglutinin, whereas in the case of absorption with platelets, though this is incomplete, it has resulted in the reduction of titer for both antigens to about the same extent. In other words the red cell is behaving

as an heterologous antigen, whilst the platelet behaves as an homologous antigen. Recalling the earlier work on anti-platelet sera, already referred to in this communication, it will be seen that these findings are in complete agreement with those of Sacerdotti, but do not support the later work of Aynaud. How are these contradictory findings to be accounted for? This at first sight appears difficult, but when one considers that the specificity of absorption reactions is relative and not absolute, an explanation is forthcoming. The strength of the serum, the dilution at which it is absorbed, the quantity of antigen employed in absorbing the serum, all these factors have to be considered. It is quite conceivable, therefore, that in absorbing a serum of low titer at comparatively high dilutions and with large doses of an heterologous antigen, both the homologous and heterologous antibodies would be removed, whereas absorption of the same serum, but at lower dilutions and with smaller doses of the same antigen, would result in the removal of the heterologous antibody only. Since the majority of these investigations have been carried out with anti-platelet sera of low titer, it would appear that the above explanation might account for the discordant results. The serological specificity of anti-platelet sera is also supported by the experimental work in animals already referred to (Ledingham and Bedson), the property of producing purpura being possessed by anti-platelet serum alone. The findings obtained with the anti-leucocyte serum (polymorph) are not without interest. It has been maintained by some investigators (Martelli (1915¹⁴) most recently) that the platelets originate from the leucocyte by a casting-off of cytoplasmic fragments. The serological specificity of this serum, as far as platelets and leucocytes are concerned, does not lend support to this theory.

CONCLUSIONS.

1. Anti-platelet serum contains a specific antibody for platelets.
2. This finding is in support of the view first put forward by Bizzozero, that the platelet is an independent element of the blood.
3. An anti-leucocyte (polymorph) serum is unable to agglutinate platelets (same species), and similarly an anti-platelet serum is without effect on leucocytes.
4. The view that the platelet arises directly from the polymorphonuclear leucocyte is not supported by this finding.

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Blood-Platelet Anti-Serum, its Specificity and Role in the Experimental Production of Purpura

BY

S. PHILLIPS BEDSON, M.D., M.Sc. (DUNELM)

Assistant Bacteriologist, Lister Institute, London

BLOOD - PLATELET ANTI - SERUM, ITS SPECIFICITY AND RÔLE IN THE EXPERIMENTAL PRODUCTION OF PURPURA.*

S. PHILLIPS BEDSON, M.D., M.Sc. (Dunelm).

Assistant Bacteriologist, Lister Institute, London.

Part II.

IN a previous communication (Bedson, 1921¹), it has been shown that an anti-platelet serum contains an antibody specific for platelets, it being possible by means of absorption to remove all red-cell antibody from such a serum and to leave the specific antibody untouched. In the same communication mention was made of the investigations carried out with regard to the production of purpura in animals (Ledingham (1914²), Ledingham and Bedson (1915³)), and it will be recalled that this phenomenon was produced by anti-platelet serum alone, neither anti-red cell serum nor anti-leucocyte serum being able to do so. Now the serum with which we then worked agglutinated both platelets and red cells, the respective titres of the anti-guinea-pig platelet serum being 1 in 128 (*v. platelets*) and 1 in 2048 (*v. red cells*). *In vivo* also this serum produces hæmagglutination, this phenomenon occurring simultaneously with the onset of purpura. In view of these observations it was thought that most probably the hæmagglutinin present in anti-platelet serum played an important rôle in the production of the hæmorrhages, the masses of agglutinated red cells held up in the capillaries leading to their rupture. Obviously this was not the only factor concerned, since an anti-red-cell serum, though capable of giving rise to hæmagglutination *in vivo*, did not produce purpura. Since it has been shown possible to remove the red-cell antibody from an anti-platelet serum, leaving untouched the platelet antibody, it was decided to test such an absorbed serum *in vivo*. The interest of this experiment is obvious. It should give us a clearer insight into the mechanism of the hæmorrhage, which up to now has remained a matter of pure conjecture, and incidentally it should throw further light on the functions of the platelet.

IN VIVO EFFECTS OF SERA PREPARED AGAINST BLOOD ELEMENTS OF GUINEA-PIG.

It was decided first of all to test *in vivo* the various sera, prepared against the blood elements of the guinea-pig, making observations at intervals on the blood picture, particularly with reference to the platelets, and noting also the presence or absence of hæmorrhages.

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This was a repetition of our earlier work, at any rate as far as the anti-platelet, anti-red cell, and anti-leucocyte sera were concerned. Finally, the absorbed anti-platelet serum was to be tested *in vivo*, noting particularly its power to give rise to hæmorrhages.

Technique of the Platelet Count.

The number of red cells and leucocytes was estimated by means of the Thoma-Zeiss pipettes and counting chamber. The following method was used in counting platelets. The ear of the guinea-pig was cleaned up with ether and a large drop of 2 per cent. citrate in saline deposited over a suitable vein. The vein was then opened *through* the drop of diluent by means of a sharp needle, the blood flowing directly into the diluent. With a platinum loop of convenient size a drop of this citrated blood was mixed on a slide with an equal quantity of a solution of brilliant cresyl blue (1 per cent. aq. sol. brilliant cresyl blue 1 part, 2 per cent. citrate in saline 6 parts) covered with a cover slip and the preparation ringed with paraffin. A sufficient quantity should be taken to spread out evenly under the cover slip without causing it to float. A satisfactory preparation must show no clumping of the platelets. The platelets (stained blue) and the red cells were then counted in a number of fields of this preparation and the ratio of platelets to red cells calculated. The number of red cells per cubic millimetre having been estimated in the usual manner, the absolute number of platelets per cubic millimetre is readily arrived at.

Experimental Data.

The findings in the guinea-pigs inoculated with the various anti-sera are tabulated below:—

TABLE I.
Anti-platelet Serum.

Time of Observation.	Red Cells.	Platelets.	Platelet/Red Cell Ratio.
Before inoculation	6,496,000	1,160,000	1 to 5·6
1·0 c.c. <i>Anti-platelet Serum</i> inoculated intraperitoneally.			
Six hours after inoculation	6,638,000	57,000	1 to 111

Animal killed with chloroform six and a quarter hours after inoculation.

Post-mortem Findings.—Skin and subcutaneous tissue show a few hæmorrhages: one or two small hæmorrhages in muscles of thigh.

Peritoneal Cavity.—Hæmorrhages in mesentery and in both large and small intestine: small hæmorrhages in epididymis. Liver and spleen appear normal: no macroscopic change in kidney or suprarenal.

Pleural Cavity.—Lungs show a few hæmorrhages: otherwise normal.

TABLE II.
Anti-red-cell Serum.

Time of Observation.	Red Cells.	Platelets.	Platelet/Red Cell Ratio.	Leucocytes.
Before inoculation	5,456,000	900,000	6·0	9,400
1·0 c.c. <i>Anti-red-cell Serum</i> inoculated intraperitoneally.				
Five hours after inoculation	5,560,000	1,010,000	5·5	20,000

Five hours after inoculation hæmagglutination was present. There were no skin hæmorrhages and bleeding time was normal. The animal died during the night.

Post-mortem Findings.—No hæmorrhages in skin or subcutaneous tissue.

Peritoneal Cavity.—Contains some blood-stained fluid : liver enlarged : gall bladder greatly distended with thin reddish bile : spleen enlarged and dark purple in colour : bladder contains blood-stained urine : kidney and suprarenals appear normal : no hæmorrhages anywhere.

Pleural Cavity.—Lungs congested, no hæmorrhages : heart contains laked blood.

TABLE III.
Anti-leucocyte Serum.

Time of Observation.	Red Cells.	Plate-lets.	Plate-let/Red Cell Ratio.	Leuco-cytes.	Differential.					Remarks.
					Polymorph.	Eosinophile.	Lymphocyte.	Mononuclear.	Mast Cell.	
Before inoculation . 1.0 c.c. <i>Anti-leucocyte (polymorphonuclear) Serum inoculated intraperitoneally.</i>	5,520,000	745,000	7.4	4600	51.3	4.0	38.0	5.3	1.3	
Four hours after inoculation	5,920,000	730,000	8.1	1000	14.0	...	73.0	12.0	1.0	No skin hæmorrhages Degenerate polymorphs in blood smear.
Twenty - four hours after inoculation	4,336,000	522,000	8.3	2400	0.5	1.0	92.5	6.0	...	No skin hæmorrhages. Hæmagglut. + Bleeding time normal.

Animal killed with chloroform after 24-hour observation.

Post-mortem Findings.—No hæmorrhages in skin or subcutaneous tissue.

Peritoneal Cavity.—Congestion of all viscera : no hæmorrhages : hæmagglutination in mesenteric vessels very marked : spleen somewhat enlarged and dark in colour : liver shows no macroscopic change.

Pleural Cavity.—Lungs, no hæmorrhages. Heart, contains agglutinated blood.

TABLE IV.
Anti-serum (precipitating) Serum.

Time of Observation.	Red Cells.	Plate-lets.	Platelet/Red Cell Ratio.	Leuco-cytes.	Remarks.
Before inoculation . . .	5,440,000	850,000	6.4	7400	
1.0 c.c. <i>Anti-serum (precipitating) Serum inoculated intraperitoneally.</i>					
Five hours after inoculation	5,200,000	800,000	6.5	7800	Bleeding time,--normal. No hæmagglutination. No purpura.
Twenty-four hours after inoculation	5,440,000	891,000	6.1	9220	No change.

TABLE V.
Anti-whole-blood Serum.

Time of Observation.	Red Cells.	Platelets.	Platelet/Red Cell Ratio.	Leucocytes.	Differential.				
					Polymorph.	Eosinophile.	Lymphocyte.	Mononuclear.	Mast Cell.
Before inoculation .	5,920,000	768,000	7·7	8,400	47·0	...	42·0	10·0	1·0
1·0 c.c. <i>Anti-whole-blood Serum inoculated intraperitoneally.</i>									
Fifteen hours after inoculation	640,000	66,000	9·6	40,000	66·0	2·5	20·5	11·0	...

At the 15-hour observation—Hæmagglutination +, bleeding time +, blood smears show active phagocytosis of red cells, nucleated red cells and myeloblasts present. Animal thereafter killed with chloroform.

Post-mortem Findings.—One or two small hæmorrhages in skin.

Peritoneal Cavity.—No excess of fluid: no free blood: liver, enlarged, firm, and has mottled appearance with yellowish areas varying in diameter from 1 to 4 mm. Spleen enlarged and deep purple in colour.

Stomach and intestine both show some punctiform hæmorrhages. Kidney and suprarenals appear normal. Bladder distended with deeply blood-stained urine.

Pleural Cavity.—No gross change noted.

It will be seen that only in the case of the anti-platelet and anti-whole-blood sera did any appreciable drop in the platelet count occur, and that these two sera alone gave rise to purpura in the guinea-pig. In the case of the anti-whole-blood serum the number of hæmorrhages was much smaller than in the case of the anti-platelet serum. Both of these sera gave rise to hæmagglutination. There are several features of interest in the findings obtained with the former serum (anti-whole-blood). The intense destruction of red cells, with a corresponding drop in the number of platelets, is seen to occur side by side with a leucocytosis of 40,000. Blood smears made at this period showed active phagocytosis of red cells both by large mononuclear and polymorphonuclear leucocytes, particularly by the former cells, many of which were literally packed with red cells. The presence of many nucleated red cells, both normoblasts and megaloblasts, and of primitive white cells of the "lymphoidocyte" type, bore evidence of a reaction on the part of the bone marrow. Occasional cells of the latter type were seen undergoing mitotic division. Whether this leucocytosis was only part of the general bone-marrow reaction or due also to the call for phagocytes to remove the damaged red cells, it is difficult to say. The specificity of an anti-leucocyte serum when tested *in vivo* is well brought out by the experiment recorded in Table III. The platelets and red cells have fallen slightly in twenty-four hours, whereas the total leucocyte count shows a 75 per cent. reduction in

Two guinea-pigs were then taken. The one, B57, was inoculated intraperitoneally with the absorbed anti-platelet serum, the other with the diluted unabsorbed serum. Blood counts were made before inoculation, four and twenty-four hours after. The two animals were then killed and examined. The findings are recorded in Table VII.

TABLE VII.

Time of Observation.	Guinea-pig B57.			Guinea-pig B58 (Control).		
	Red Cells.	Plates.	Platelet /Red Cell Ratio.	Red Cells.	Plates.	Platelet /Red Cell Ratio.
*Before inoculation . . .	6,096,000	836,000	7·3	5,760,000	789,000	7·3
Five hours after . . .	5,712,000	111,000	49·3	5,536,000	36,000	153·6
Twenty-four hours after . .	3,648,000	35,000	102·4	3,920,000	55,000	70·6
	<i>Post-mortem Findings.</i>					
	Skin and sub-cut. tissue . .	Numerous hæmorrhages .			Numerous hæmorrhages.	
	Lymph glands . . .	Deep red in colour . . .			As in B57.	
	Peritoneal cavity . . .	Contains some free blood			Very little free blood.	
	Stomach and intestines . .	Numerous hæmorrhages .			Numerous hæmorrhages.	
	Spleen and liver . . .	Nil			Nil.	
	Pleural cavity . . .	Contains a good quantity of free blood			No free blood.	
	Lungs	Nil			Several large hæmorrhages.	
Heart	<i>Heart blood shows no agglutination</i>			<i>Heart blood shows well-marked agglutination.</i>		
Bleeding time in both animals lengthened considerably.						

* B57 received 4.0 c.c. "absorbed anti-platelet serum" (represents 1.0 c.c. undiluted serum) intraperitoneally.

B58 received 4.0 c.c. unabsorbed anti-platelet serum (diluted 1/4) intraperitoneally.

In both guinea-pigs the platelets have been almost entirely removed from the circulation, this diminution preceding the less extensive fall in the red count. No hæmagglutination was noted in the guinea-pig receiving the absorbed anti-platelet serum, whilst in the control animal this phenomenon was well developed. Both animals developed severe purpura. It was therefore obvious from this experiment that the red cell agglutinin present in anti-platelet serum played no part in the production of the hæmorrhages. What then are the factors concerned? Is the removal of the platelet from the circulation sufficient of itself to give rise to the hæmorrhages? It was a simple matter to test the correctness of this hypothesis, as it has been demonstrated by many workers (Aynaud (1911⁵), Roskam (1921⁶)) that the intravenous inoculation of such substances as peptone or Bordet's "anaphylatoxin" bring about a considerable though very transitory fall in the platelet count. The effect of "agar-serum" administered intravenously was therefore tried. The "agar-serum" was prepared by

absorbing fresh normal rabbit serum with 0·5 per cent. agar in normal saline (8·0 c.c. serum to 2·0 c.c. agar saline). This mixture was placed in the incubator at 37°C. for two hours and then allowed to stand at room temperature overnight, when the agar was removed by centrifugalisation. Small rabbits (800-1000 grms.) receiving 3·0-5·0 c.c. of this agar-serum intravenously showed a considerable though transitory fall in the platelet count. On no occasion was this accompanied by the production of hæmorrhages. The observations made on one of these rabbits are given in Table VIII.

TABLE VIII.

Time of Observation.	Red Cells.	Platelets.	Platelets / Red Cell Ratio.	Remarks.
*Before inoculation . . .	5,200,000	702,000	7·4	Some polypncea after inoc. Soon recovered.
Thirty minutes after	123,000	41·2	
One hour after	5,160,000	521,000	9·9	
One and a half hours after	Killed (chloroform)	and examined.		

* 3·0 c.c. "agar-serum" inoculated intravenously.

Post-mortem Findings.—Mesenteric vessels engorged : no hæmorrhages observed.

Since the removal of platelets from the circulation alone is unable to give rise to purpura, there must be additional factors concerned in the production of this condition. The mechanical factor (hæmagglutination) has been ruled out. No obvious change in the other blood elements has been brought to light which might help to explain the hæmorrhages, though Musser and Krumbhaar (1916⁷) consider the increased fragility of the red cell, which they find in experimental purpura, as being an essential factor. It is difficult to see why this should be. It has long been thought that some damage to the endothelium of the capillaries was essential to the production of purpura, and this is borne out by an examination of the tissues from guinea-pigs which have received inoculations of anti-platelet serum. The endothelium appears swollen and œdematous, the cells standing off the vessel wall. Supposing this to be the other factor concerned in the genesis of the hæmorrhage, then it should be supplied by any of the sera prepared against the blood elements, since they are all more or less closely related genetically to the endothelial cell. Assuming this to be correct, we then have two factors to consider, viz., the damage to the capillary endothelium and the removal of the platelet from the circulation. It has been already seen that anti-red-cell and anti-leucocyte sera are unable to give rise to purpura, presumably because the second factor is absent. It is in anti-platelet serum only that we find these two factors combined. If, however, an animal were inoculated first of all with an anti-red-cell serum and after a

suitable interval to allow for action on the endothelium of the vessels the platelets were removed, even temporarily, from the circulation, then we should have present in that animal the conditions necessary for the production of hæmorrhage and purpura should result.

MECHANISM OF THE HÆMORRHAGE.

To test this hypothesis the following experiment was carried out. A small rabbit (900 grms.) was inoculated with 0·75 c.c. anti-rabbit-red-cell serum intravenously. The red cells and the platelets were counted before and four and a half hours after inoculation. It then received 4·5 c.c. "agar-serum" intravenously, and the platelets and red cells were again counted after the lapse of thirty minutes. One hour after the second inoculation the animal was killed with chloroform and examined. *The post-mortem findings were similar to those produced by an anti-platelet serum.* The details of this experiment are summarised in Table IX.

TABLE IX.

Time of Observation.	Red Cells.	Platelets.	Platelet /Red Cell Ratio.	Remarks.
* Before inoculation .	4,800,000	631,000	7·6	Rabbit very collapsed. Breathing embarrassed. Passed some blood-stained urine.
3.0 p.m.	4,200,000	558,000	7·7	
† 3.50 p.m.	3,680,000	92,000	39·9	
4.10 p.m.	Killed (chloroform)		and examined.	

* 0·75 c.c. anti-rabbit-red-cell serum inoculated intravenously 10.30 a.m.

† 4·5 c.c. "agar-serum" inoculated intravenously 3.10 p.m.

Post-mortem Findings.—Skin and subcutaneous tissues show a number of small hæmorrhages particularly in loose tissue over back.

Peritoneal Cavity contains some free, blood-stained fluid and one or two blood clots of moderate size: great omentum shows some small hæmorrhages: pancreas, one or two petechial hæmorrhages: intestine, numerous small punctiform hæmorrhages, particularly in lower portion of descending colon: kidneys and suprarenals, no macroscopic change: bladder, full of blood-stained urine: urine contains some unchanged red cells and granular casts: liver drips with blood on section—surface has a "mottled" appearance: spleen slightly enlarged and of deep purple colour.

Pleural Cavity.—Numerous hæmorrhages along the course of intercostal vessels: lungs show numerous hæmorrhages about 2 mm. in diameter.

This experiment was repeated with a precisely similar result. It has been shown that the "agar-serum" alone was unable to produce this pathological picture, and anti-red-cell sera, when tested in the guinea-pig, did not reduce the platelet count appreciably nor give rise to purpura. The effect of an anti-red-cell serum alone was further controlled by inoculating rabbits with one intravenously. This was done on two occasions and in neither case were any hæmorrhages produced. The details of one of these control experiments are given in Table X.

TABLE X.

Time of Observation.	Red Cells.	Platelets.	Platelet /Red Cell Ratio.	Remarks.
Before inoculation .	4,240,000	588,000	7.2	
0.75 <i>Anti-rabbit-red-cell Serum inoculated intravenously.</i>				
Five and a half hours after	3,880,000	562,000	6.9	Animal somewhat collapsed after inoculation, but had recovered in thirty minutes.

Animal killed (chloroform) and examined.

Post-mortem Findings.—Skin and subcutaneous tissue: no hæmorrhages.

Peritoneal Cavity.—Vessels of mesentery engorged, no hæmorrhages: spleen, slightly enlarged, deep purple in colour: liver, kidney, and suprarenals appear unchanged: bladder contains blood-stained urine.

Pleural Cavity.—Nothing abnormal noted: no hæmorrhages.

Similarly no hæmorrhages are produced when the platelets are removed from the circulation of a rabbit which has received four and a half hours previously a normal foreign serum.

DISCUSSION.

It has been shown then that anti-platelet serum when inoculated into the animal for which it is specific produces purpura, whereas the sera prepared against the other blood elements—red cells, leucocytes, serum—do not. It is now well established that the hæmorrhagic diathesis in man is associated with a low platelet count, this tendency being the most severe in those cases where the platelets have almost entirely disappeared from the circulating blood. The destruction of the platelet or its removal from the circulating blood (for we are not certain of its fate) is brought about by anti-platelet serum. The other anti-sera do not produce this change. Obviously this is one of the factors concerned in the production of purpura. That it is not the only one is shown by the experiments with “agar-serum.” Although by this means the platelet counts can be very considerably diminished, hæmorrhages do not occur. In a recent paper by Roskam (already referred to), attention is drawn to the fact that in animals in which a reduction of the platelet count had been produced by the intravenous injection of a solution of gelatine, the bleeding time was only slightly prolonged. Now an increased bleeding time is a constant feature of purpura. Roskam therefore concludes that there must be some other change occurring in this condition, most probably some damage to the capillary endothelium. First proposed by Nolf, this view was also held by Ledingham, and a histological examination of the tissues from purpuric animals supports this contention, as change in the vascular endothelium, similar to those described by Findlay

(1921⁸) in experimental scurvy, can be made out, namely, a swollen and œdematous condition of these cells. It might be argued that this swelling of the capillary endothelium, with the consequent narrowing of the lumen of the vessel, would bring about a slowing of the circulating blood. The platelets would then adhere to the altered endothelial cells, leading eventually to a plugging of the vessel and its ultimate rupture. No evidence, however, has been found to support this view. Further, if a guinea-pig is given an inoculation of anti-platelet serum, and one to two hours later it is anæsthetised and its mesenteric vessels observed under the microscope, it is seen that the hæmorrhages form along the course of the vessels in which the circulatory current is not appreciably slowed. No evidence of plugging of the capillaries is to be seen, and, as far as one can see, the majority of the hæmorrhages appear to form gradually at different points along the course of the vessel, by what appears to be an exaggerated form of diapedesis. It would seem that in purpura, produced by means of an anti-platelet serum, the sequence of events in the production of hæmorrhages is as follows: the capillary endothelium is first of all damaged, and later, when the platelets have been greatly reduced in number in the circulating blood, a leakage of red cells through the capillary wall into the surrounding tissues takes place. Had the platelets been present hæmorrhages would not have occurred, because their presence in the outer more slowly moving stream, together with the leucocytes, would have prevented the rapidly moving central stream of red cells from coming in contact with the endothelium and of finding out its weak spots. They would also have helped in making good the deficiencies in the vessel wall caused by the endothelial damage.

CONCLUSIONS.

1. Of various anti-sera prepared by immunisation with blood elements (cellular and otherwise), anti-platelet serum alone produces purpura.

2. This purpura is the result of the action of platelet antibody alone and takes place independently of any hæmagglutination.

3. An extensive, though temporary, reduction in the number of platelets in the circulating blood of the rabbit does not give rise to purpura.

4. Experimental evidence has been brought forward to show that the two main factors concerned in the production of the hæmorrhages are:—

(a) Toxic action on the endothelium of the vessels.

(b) Removal of the platelets from the circulation.

5. The serological specificity of anti-leucocyte serum, as far as blood cells are concerned, is paralleled by its specificity when tested *in vivo*.

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No 9

THE BLOOD PICTURE IN SCURVY, WITH PARTICULAR REFERENCE TO THE PLATELET.

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BY

S. PHILLIPS BEDSON, M.D., M.Sc. DUNELM.,
Assistant Bacteriologist, Lister Institute, London.

It is now generally accepted that in those pathological conditions which are accompanied by a diminution in the number of blood platelets a tendency to haemorrhage is almost always observed.

Already in 1910 Duke,¹ from the analysis of a large number of platelet estimations in different clinical conditions, arrived at the following conclusions: (1) When the platelet count descends below the level of 60,000 per c.mm. there is an abnormal tendency to bleed; (2) if the count falls below 10,000 this tendency is always present; and (3) when below 1,000 it is present in its most severe form. The production of experimental purpura in animals (Ledingham,² 1914) by means of an anti-platelet serum, and following on this the observation that anti-platelet serums alone of the antiblood-element serums were capable of producing this pathological picture (Ledingham and Bedson, 1915³), completed the chain of evidence.

In scurvy we have another clinical condition in which haemorrhage is a constant feature. The bleeding from the gums and the subperiosteal haemorrhages over the tibiae, the "black eye," these are all characteristic signs met with in the acute stages of the disease. In experimental scurvy in the guinea-pig the haemorrhages are sometimes so numerous as to recall vividly the picture produced in these animals by means of an anti-platelet serum. The question, therefore, naturally arose as to whether or not the platelets were reduced in numbers in the acute stages of scurvy. On searching through the literature the observations made on this point were found to be extremely scanty.

S. Wassermann⁴ (1918), in a communication on the blood picture in scurvy as it occurs in man, remarks that the blood platelets are reduced in number in the acute stage, and increased in convalescence. Kirch⁵ (1919) gives the findings in two cases, the platelet counts being 280,000 and 14,000 per cubic millimetre, corroborating the observations of Wassermann. However, no details of the technique used by these two workers in the counting of platelets is given, so that it is more or less impossible to judge of the accuracy of their observations. Furthermore, in the two cases of Kirch's, bleeding times of 3 and 2.7 minutes respectively are given, which makes his platelet counts even more difficult to accept. Hess⁶ (1914), in a communication on infantile scurvy, states that the platelet count is normal and that the haemorrhages are most probably due to a decreased capillary resistance. In view of these contradictory findings it was decided to work out the picture in scurvy experimentally produced in guinea-pigs, paying particular attention to the platelet count.

Technique.

In the counting of red cells and leucocytes, and in making the differential leucocyte count, the usual technique was employed. The haemoglobin estimations were made by means of Haldane's modification of Gowers's haemoglobinometer. The number of platelets was estimated by the following method :

The skin (of the ear in most cases) is cleaned up with ether, and on the clean surface is deposited a large drop of diluent (the diluent employed consisted of 1 per cent. aqueous solution of brilliant cresyl blue 1 part, 2 per cent. sodium citrate in normal saline 6 parts). The skin is then stabbed, through the drop of diluent, by means of a sharp needle. The blood thus oozes directly into the diluent, which prevents clumping of the platelets, and at the same time stains them blue. With a platinum loop of convenient size some of the diluted blood is transferred to a slide and carefully covered with a cover-slip. The amount of blood taken should be sufficient to spread out evenly between the slide and cover-slip without causing the latter to float. The preparation is ringed with vaseline. The number of platelets and red cells is then counted in several fields of the preparation (Leitz 7, ocular 3), and the ratio of platelets to red cells determined. Having already determined the number of red cells per cubic millimetre, the actual number of platelets is readily arrived at.

Experimental Data.

A series of four guinea-pigs was put on a diet of bran, oats, and water only (no green food or autoclaved milk), it having been shown that such a diet produces acute scurvy with death in the guinea-pig in about three weeks' time (H. Chick, E. M. Hume, and R. F. Skelton⁷). Blood estimations were made before the commencement of the experiment and at intervals of three to four days during its course.

These guinea-pigs lived for periods ranging from twenty-six to thirty-one days on the scurvy diet, and also show an appreciable loss in weight, roughly one-third of their original weight. *Post-mortem* the findings characteristic

of scurvy were noted, though it must be admitted that these were not as severe as they usually are. The teeth were loose, there was some "beading" of the junction of the costal cartilages with the true ribs. Both small and

TABLE I.

Date. 1921.	Weight Grams	Red Cells.	Leuco- cytes.	Platelets.	Remarks.
GUINEA-PIG B. 41.					
Jan. 24 ...	—	4,864,000	5,600	600,000	
" 27* ...	295	5,168,000	4,000	781,000	
" 31 ...	320	5,920,000	6,600	883,000	
Feb. 3 ...	330	6,496,000	8,200	713,000	
" 7 ...	280	7,888,000	5,000	1,655,000	Not lively; coat rough.
" 10 ...	270	6,566,000	4,400	1,042,000	Unchanged.
" 14 ...	280	6,896,000	4,000	1,275,000	"
" 17* ...	225	5,536,000	4,600	748,000	"
" 19 ...	210	—	—	—	In moribund state.
GUINEA-PIG B. 42.					
Jan. 24 ...	—	5,470,000	7,000	824,000	
" 27* ...	325	6,066,000	11,200	797,000	
" 31 ...	310	6,928,000	10,200	1,330,000	
Feb. 3 ...	330	6,896,000	9,400	1,300,000	
" 7 ...	270	7,584,000	6,400	1,547,000	
" 10 ...	290	6,272,000	12,800	896,000	
" 14 ...	300	6,480,000	11,600	841,000	
" 17 ...	260	5,024,000	9,000	913,000	Quiet; out of condition.
" 19 ...	270	5,472,000	6,400	781,000	
" 21 ...	260	5,536,000	6,000	779,000	Looks ill.
" 22 ...	250	5,344,000	—	785,000	Unchanged.
" 23 ...	240	4,960,000	—	82,000	"
" 24 ...	220	—	—	—	In dying condition.
GUINEA-PIG B. 43.					
Jan. 24 ...	—	5,766,000	10,600	784,000	
" 27* ...	280	5,152,000	5,000	792,000	
" 31 ...	300	7,986,000	6,600	1,477,000	
Feb. 3 ...	320	6,368,000	7,200	936,000	
" 7 ...	260	7,072,000	4,800	1,105,000	
" 10 ...	300	5,808,000	6,200	735,000	
" 14 ...	270	5,504,000	6,000	797,000	
" 17 ...	225	6,016,000	4,800	761,000	Quiet; out of condition.
" 19 ...	200	4,848,000	7,200	673,000	Very ill.
" 20 ...	—	—	—	—	Died during night.
GUINEA-PIG B. 44.					
Jan. 24 ...	—	5,680,000	8,000	800,000	
" 27* ...	430	6,144,000	6,000	739,000	
" 31 ...	410	6,864,000	4,300	1,183,000	
Feb. 3 ...	410	6,624,000	11,000	973,000	
" 7 ...	340	8,000,000	5,400	754,000	
" 10 ...	390	5,968,000	7,000	575,000	
" 14 ...	360	6,352,000	4,600	907,000	
" 17 ...	325	5,648,000	3,600	910,000	Looks ill.
" 19 ...	300	6,208,000	6,000	886,000	Unchanged.
" 21 ...	270	5,200,000	6,800	675,000	Very ill.
" 22 ...	—	—	—	—	Died 10 a.m.

* Put on diet of bran, oats, and water *ad lib.*

Differential Leucocyte Count.—This showed fluctuations such as might be met with in normal animals—no constant or striking change. No lymphocytosis, relative or absolute, was noted.

Post mortem Findings.—Guinea-pig B. 41: Some swelling of costo-cartilaginous junctions; small haemorrhages in intestines; suprarenals congested, small haemorrhages. Guinea-pig B. 42: Small haemorrhages in gums; swelling of costo-cartilaginous junctions; large intestine shows one or two haemorrhages ($\frac{1}{2}$ in. in diameter); haemorrhages in thigh muscles. Guinea-pig B. 43: Swelling of costo-cartilaginous junctions well marked; haemorrhages in both large and small intestine; suprarenals show some petechial haemorrhages. Guinea-pig B. 44: Swelling of costo-cartilaginous junctions; small haemorrhages in intestines.

large intestine showed some small haemorrhages, and the suprarenals were congested and showed some small petechial haemorrhages. The bone marrow of the femur was of the red active type.

As regards the blood picture, no change of an outstanding character has occurred. The red cells are apparently increased in what we might term the "prescurvy" period, dropping again to normal count at death. The platelets show the same increase and subsequent decrease. Even shortly before their death, when the animals were very weak, the platelets were normal in number. The total and differential leucocyte counts show nothing worthy of note. These findings, it must be admitted, were somewhat unexpected, as a drop in the platelet count was confidently anticipated. However, about this time, through the kindness of my colleague, Dr. Harden, I was able to make some platelet counts on monkeys suffering from experimental scurvy, which bore out the results obtained in the guinea-pig.

TABLE II.—*Platelet Count of Monkeys suffering from Scurvy.*

Date.	Red Cells.	Platelets.	Platelet Ratio.	Remarks.
MONKEY I. Feb. 24, 1921	4,208,000	576,000	7.3	Gums fungating; some bleeding. Teeth loose. Avoids use of hind legs (subperiosteal haemorrhages). Pallor of face.
Mar. 8, 1921	4,352,000	800,000	5.5	Condition more severe. Proptosis of right eye.
MONKEY II. Feb. 24, 1921	5,737,000	735,000	7.8	Condition not so acute as in Monkey I. Few haemorrhages to be seen in gums.
MONKEY III.* Feb. 26, 1921	4,560,000	600,000	7.6	

* Monkey III was the normal control animal.

Both these monkeys, though showing the typical picture of acute scurvy, gave more or less normal platelet counts. In Monkey I, in which the condition was the most acute, the red cells show a slight drop, whilst in Monkey II the red count is above that obtained in the normal control. Wassermann, in the paper already referred to, states that the red cells are sometimes slightly below the normal

number, whilst in other cases a high red count up to seven millions is obtained. It is difficult to understand why an increase in the number of red cells should be met with in this disease, unless it is due to a diminution in the total volume of the blood. If this were so, the normal total leucocyte counts noted in the case of the scorbutic guinea-pigs would in reality represent a leucopenia.

In conclusion, the observations made in the case of two infants might be of interest, as pointing to a practical application of these experimental findings, and at the same time further supporting the contention that the platelets are not affected in scurvy. It is through the kindness of Dr. D. H. Paterson, of the Children's Hospital, Great Ormond Street, that I have had access to this material and am able now to publish these figures. They are tabulated below (see Table III).

In both these cases a diagnosis of scurvy had been made, though it must be admitted that in case R. E. there was some doubt as to the correctness of this view. When, however, the additional evidence afforded by the platelet count was available it was at once seen that the two cases were essentially deficient. In R. E. the platelet count was

TABLE III.

Case.	Age.	Bleeding Time.	Red Cells.	Platelets.	Plate Ratio.	Blood Smear.
R. E.	1½	++	3,248,000	60,000	53.3	Nucleated red cells. Polychromatophilia. Anisocytosis.
F. S.	10/12	Normal	4,000,000	560,000	6.8	Nothing abnormal seen.

Note.—R. E. had haemorrhages (skin) scattered all over the body bleeding from mucous surfaces; subperiosteal haemorrhages (tibiae); a bruise 1 in. in diameter over the right eye. F. S. had bleeding from the gums, subperiosteal haemorrhages, and proptosis of both eyes.

found to be very low, and this fact, taken in conjunction with the low red count and evidence of a bone marrow reaction, confirmed the doubts felt as to the original diagnosis of scurvy, and it was changed to one of purpura haemorrhagica. The second case, F. S., in which there was little doubt as to the true condition, gave a normal platelet count and a red count slightly below the normal. The subsequent history of these cases bore out the revised diagnosis. Both children received antiscorbutic treatment. R. E. died in the course of a few days, with *post-mortem* findings in keeping with the diagnosis of purpura haemorrhagica, whereas F. S. made a rapid recovery.

Conclusions.

1. In scurvy produced experimentally in guinea-pigs and monkeys and in one human case the platelets were found to be normal in number. It is possible that very transient fluctuations in the platelet count occur in scurvy,

and that it is during this period of platelet deficiency that the haemorrhages occur. It is hardly conceivable, however, that in making a comparatively large number of platelet observations such fluctuations should have been completely overlooked. Demmer⁸ cites a case of purpura haemorrhagica in which fluctuations in the platelet count, preceding the outburst of haemorrhages, occurred in a most regular manner.

2. The red cells in some cases showed an increase in number, this condition coinciding with a "prescurvy" or incipient scurvy stage. In the acute stages of the disease, particularly where haemorrhages were numerous, the number of red cells fell to slightly below the normal.

3. No variations in the total and differential leucocyte count were observed.

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DYSENTERY IMMUNISATION IN RABBITS BY THE ORAL AND SUBCUTANEOUS METHODS.

S. KANAI, M.D.,

Tokyo.

From the Bacteriological Department, Lister Institute, London

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THE possibility of immunisation *per os* was first demonstrated by Ehrlich (1891), who succeeded in eliciting the development of anti-ricin in rabbits by feeding them with ricin. This question was left more or less in abeyance until 1905, when Zeitlin (1905) showed that rabbits, to which a killed suspension of *B. dysenteriae* (Shiga) had been administered orally, developed agglutinins for that organism in their serum. In view of these findings he suggested that the oral route should be employed in the prophylactic immunisation of man against such acute intestinal diseases as cholera, dysentery and enteric. Working along these lines Hida and Toyoda (1907) demonstrated the production of antibodies in guinea-pigs by the administration *per os* of dysentery bacilli (type Shiga) previously digested with pepsin and trypsin. The ingestion of dysentery bacilli, which had merely been killed by heating to 60° C., was, according to these workers, unable to give rise to any antibody response, but Shiga (1908) claims to have immunised rabbits against this same organism by the oral administration of heat-killed vaccines.

More recently Besredka (1919) brings forward experimental evidence in support of oral immunisation. He claims that a solid immunity, as tested by intravenous injection of the live organism, can be produced in rabbits against *B. dysenteriae* (Shiga), *B. paratyphosus* B. and *B. typhosus* by the introduction *per os* of killed vaccines. Rabbits immunised in this manner were able to support the intravenous inoculation of otherwise lethal doses of living organisms. This method of immunisation was found by him to be more efficacious in the cases of *B. paratyphosus* and *B. typhosus* if the vaccine was mixed with a certain quantity of sterile bile. From his observations Besredka concluded that the intestinal mucosa forms the first line of defence against

invasion by these organisms—or the toxic products of these organisms—which give rise to acute intestinal conditions. This contention is supported by the observation that the first ingestion of antigen gave rise to the appearance of antibody in the blood, whereas the subsequent ones did not; in fact, at that period where he was able to demonstrate immunity in his animals, the agglutinins had practically disappeared. This immunity he therefore considers to be mainly local—an increased resistance on the part of the cells of the intestinal mucous membrane to the passage of the infecting organism or of its toxin—the damaged cells which are cast off in the catarrhal condition set up by the infection being replaced by new ones possessed of this acquired property. Besredka looks upon this initial catarrhal stage as essential to the establishment of a satisfactory local immunity, and for this reason bile is administered with the vaccine.

In appraising the practical value of any method of prophylactic immunisation we should consider the following points:

- (I) The degree of immunity obtained.
- (II) The intensity of reaction, local and general, produced by the vaccine.
- (III) The simplicity of administration.

It is generally recognised that the local and general reactions following the subcutaneous inoculation of Shiga vaccines in all but low doses are often intense. In view, therefore, of Besredka's finding that a solid immunity may result from the oral administration of the vaccine, it was decided to carry out experiments in rabbits with the object of determining the degree of such resistance to *B. dysenteriae* (Shiga), and to compare it with the immunity resulting from the usual subcutaneous inoculation.

SCHEME OF RESEARCH.

First Series of Experiments.

Two strains of *B. dysenteriae* (Shiga) were employed in this investigation. One came from the National Collection of Type Cultures (No. 151), and for the other I am indebted to the kindness of Dr. Besredka. Both these strains were typical, culturally and serologically. The work reported in this communication was carried out with strain No. 151 practically entirely.

Ingestion of vaccines.—The 24-hour growth on agar obtained from one Roux bottle was suspended in 20 c.c. of sterile normal saline and heated for one hour at 60° C. Measured quantities of this suspension were mixed with bran and fed to rabbits which had fasted for 24 hours.

Antibody production (agglutinins).—Test bleedings were made before, and at intervals during the immunisation. The titre of antibody was determined by means of the agglutination reaction, results being read after four hours' incubation at 37° C. and again after standing at room temperature for twenty-four hours.

Degree of immunity produced.—This was determined by the intravenous inoculation of lethal doses of living *B. dysenteriae* (Shiga), the virulence of which for the rabbit had been previously determined.

EXPERIMENTAL DATA.

The Virulence of Strain No. 151.

A saline suspension of a 24-hour agar slope culture was inoculated intravenously in a series of four rabbits, the dose varying from 50×10^6 to 500×10^6 . The results are recorded in Table I:

TABLE I.

No. of rabbit.	Weight.	Number of bacilli inoculated.	Result.
17 .	1740 grm.	500×10^6	Died 2 days after injection.
18 .	1535 „	250×10^6	„ 8 „ „ „
19 .	1700 „	100×10^6	Survived.
20 .	1450 „	50×10^6	Survived.

Post-mortem Findings.

No. 17.—Small intestine congested and mucous membrane œdematous. The organism was recovered from the intestinal contents but not from the spleen.

No. 18.—Small intestine showed the same changes as in Rabbit 17. No hæmorrhages. Large intestine and appendix appear normal. Cultures from the heart-blood, gall-bladder, spleen and intestinal contents proved sterile.

From these experiments it would appear that the M.L.D. of this strain was about 500×10^6 . In later experiments this dose was sometimes found insufficient to kill medium-sized rabbits, and accordingly 1000×10^6 was adopted as the test dose.

FIRST SERIES OF EXPERIMENTS.

Effect of Oral Immunisation.

Eight rabbits were immunised, as follows:

Nos. 5 and 6 received $\frac{1}{2}$ of a Roux bottle of killed dysentery (Shiga) bacilli.

Nos. 7 and 8 received $\frac{1}{5}$ of a Roux bottle of killed dysentery (Shiga) bacilli.

Nos. 9 and 10 received $\frac{1}{10}$ of a Roux bottle of killed dysentery (Shiga) bacilli.

Nos. 11 and 12 received $\frac{1}{5}$ of a Roux bottle of killed dysentery (Shiga) bacilli.

In the two latter (Nos. 11 and 12) the ingestion was preceded by the oral administration of 8.0 c.c. of ox bile. Of these eight rabbits two died, No. 5 on the fifteenth day after ingestion and No. 8 on the fourteenth day. No obvious cause for death was found. The remaining six rabbits were divided into two groups. Group 1 (Nos. 6, 7 and 9) were inoculated with 500×10^6 living bacilli intravenously on the nineteenth day after the ingestion of the killed dysentery bacilli. Group 2 (Nos. 10, 11 and 12) received 1000×10^6 living bacilli twenty-six days after the immunising dose. The result of the experiment is given below (Tables II and III).

TABLE II.—(Group I.)

Rabbit No. 6.

Date.	Immunising dose.	Body weight.	Agglutinin titre.	Test dose. Living <i>B. dysenteriae</i> (Shiga).	Result.
4/9/20 .	$\frac{1}{2}$ Roux bottle killed bacilli	1820 gm.	<i>Nil</i>	.	.
8/9/20 .		1680	„	.	.
11/9/20 .		1800	„	1 in 80	.
15/9/20 .		1850	„	.	.
18/9/20 .		1890	„	1 in 80	.
22/9/20 .		1960	„	.	.
23/9/20 .		.	.	500×10^6 intra-venously	Survived.

Rabbit No. 7.

4/9/20 .	$\frac{1}{5}$ Roux bottle killed bacilli	1445 gm.	<i>Nil</i>	.	.
8/9/20 .		1640	„	.	.
11/9/20 .		1640	„	1 in 40	.
15/9/20 .		1710	„	.	.
18/9/20 .		1730	„	1 in 40	.
22/9/20 .		1870	„	.	.
23/9/20 .		.	.	500×10^6 intra-venously	Survived.

Rabbit No. 9.

4/9/20 .	$\frac{1}{10}$ Roux bottle killed bacilli	1490 gm.	<i>Nil</i>	.	.
8/9/20 .		1560	„	.	.
11/9/20 .		1530	„	1 in 20	.
15/9/20 .		1530	„	.	.
18/9/20 .		1560	„	1 in 20	.
22/9/20 .		1730	„	.	.
23/9/20 .		.	.	500×10^6 intra-venously	Paralysed. Died on 6th day.

Rabbit No. 21 (Control).

23/9/20 .		1900 gm.	.	1000×10^6 intra-venously	Died on 3rd day.
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Rabbit No. 22 (Control).

23/9/20 .		1840 gm.	.	500×10^6 intra-venously	Paralysis of hind legs on 3rd day. Recovered.
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Post-mortem Findings.

No. 9.—Small intestine showed some œdema but no hæmorrhages. Large intestine appeared normal. Some hæmorrhages in appendix. Lungs, spleen, liver, kidney showed no macroscopic change.

No. 21 (control).—Some small hæmorrhages in right lung. Spleen, liver, kidney and suprarenals appear normal. Small intestine œdematous and injected. Large intestine shows no macroscopic change. Rabbit had coccidiosis.

TABLE III.—(Group II.)

Rabbit No. 10.

Date.	Immunising dose.	Body weight.	Agglutinin titre.	Test dose. Living <i>B. dysenterix</i> (Shiga).	Result.
4/9/20 .	$\frac{1}{10}$ Roux bottle killed bacilli	1380 gm.	Nil .		
8/9/20 .		1500 „	„ .		
11/9/20 .		1510 „	1 in 80 .		
15/9/20 .		1500 „	„ .		
18/9/20 .		1520 „	1 in 80 .		
22/9/20 .		1680 „	„ .		
26/9/20 .				1000×10^6 intra- venously	Paralysis of hind legs. Died 3rd day.

Rabbit No. 11.

4/9/20 .	$\frac{1}{5}$ Roux bottle killed bacilli + 8.0 c.c. ox bile	1370 gm.	Nil .		
8/9/20 .		1340 „	„ .		
11/9/20 .		1330 „	1 in 20 .		
15/9/20 .		1420 „	„ .		
18/9/20 .		1410 „	1 in 20 .		
22/9/20 .		1460 „	„ .		
26/9/20 .				1000×10^6 intra- venously	Survived.

Rabbit No. 12.

4/9/20 .	$\frac{1}{5}$ Roux bottle killed bacilli + 8.0 c.c. ox bile	980 gm.	Nil .		
8/9/20 .		860 „	„ .		
11/9/20 .		860 „	1 in 40 .		
15/9/20 .		850 „	„ .		
18/9/20 .		855 „	1 in 40 .		
22/9/20 .		930 „	„ .		
26/9/20 .				1000×10^6 intra- venously	Survived.

TABLE III.—(Group II)—*continued*.*Rabbit No. 23 (Control).*

Date.	Immunising dose.	Body weight.	Agglutinin titre.	Test dose. Living <i>B. dysenteriae</i> (Shiga).	Result.
26/9/20.		1780 grm. .		1000×10^6 intra- venously.	Died 2nd day.

Post-mortem Findings.

No. 10.—Lungs, kidney and suprarenals showed no change. Liver markedly congested. Small intestine, injection and œdema of mucous lining. Rabbit infected with coccidiosis.

No. 23.—Lungs show some small hæmorrhages. Congestion of liver, kidney and spleen. Large intestine showed no change. Small intestine injected and œdematous. No hæmorrhages to be seen macroscopically. Rabbit infected with coccidiosis.

It would appear, then, from these experiments that a certain degree of immunity to *B. dysenteriae* (Shiga) can be produced in rabbits by the oral administration of killed vaccines, but the experiment as a whole is not completely satisfactory, owing to the failure (in Group 1) of 500×10^6 to kill the control and to the complicating coccidiosis infections. The ingestion of the killed bacilli resulted in the appearance of agglutinins in the serum in every case, but the titre was never a high one. Furthermore, a perusal of these figures shows that the degree of immunity attained does not run parallel with the titre of agglutinins. It will be seen also from the body weights recorded in these two tables, that the ingestion of enormous quantities of killed dysentery bacilli is practically without effect on the general health of the animal. Before proceeding to record the second series of experiments, some observations on the effects of ingestion of living dysentery bacilli and of repeated doses of the killed organism are appended.

Effect of Ingestion of Living B. dysenteriae (Shiga) on the Rabbits.

In order to ascertain whether or not rabbits could be infected by the introduction *per os* of living bacilli, half a 24-hour Roux bottle culture of living dysentery bacilli was given to a rabbit by the mouth in one dose. The body weight, which on the day of ingestion, 11/9/20, was 1560 grm., fell to 1400 grm. on the 19th of the same month, and by 11/10/20 had still further declined to 1260 grm. Otherwise the animal appeared perfectly well. The examination of its serum for the presence of antibody (agglutinins) gave negative results throughout. Even the administration of large quantities of living dysentery bacilli to rabbits by the mouth is without effect. Similar investigations carried out by Shiga (1898) and Flexner and Sweet (1906) gave negative results also.

Result of a Series of Oral Immunisation Doses.

To determine the degree of immunity produced by a series of doses *per os*, three rabbits were taken (Nos. 2, 3 and 4) and given three doses of $\frac{1}{5}$ of a Roux bottle culture at intervals of ten days. Unfortunately rabbit No 1 died four days after the second dose from pneumonia, associated with *B. pyocyaneus*,

and rabbit No. 4 became infected with an organism of the hæmorrhagic septicæmia group, and had to be discarded. The remaining animal, No. 3 (1780 gm.) was inoculated intravenously with 2000×10^6 living *B. dysenteriae* (Shiga) on the twenty-fifth day after the last ingestion of vaccine, and at the same time a control rabbit (1700 gm.) received the same dose. The control animal died the third day after the test dose, and rabbit No. 3 became paralysed on the third day and died on the fourth. The post-mortem findings in the control rabbit were much more severe than in the immunised one. Whereas No. 3 merely showed some œdema of the small intestine with one small hæmorrhage in the appendix, the control showed injection and a more marked œdema of the intestinal mucous membrane, with numerous hæmorrhages in the ascending colon, in which was also some diphtheritic ulceration. The lungs showed some small hæmorrhages, and the liver, kidneys, suprarenals and spleen were acutely congested, the latter organ being much enlarged (three times the normal size). *B. dysenteriae* (Shiga) was isolated from the gall-bladder and spleen, but not from the contents of the large intestine. Although the post-mortem findings point to much more extensive damage in the control rabbit, it must be admitted that none of the other control animals employed in this investigation showed such marked changes. This experiment must be considered, therefore, as inconclusive.

SECOND SERIES OF EXPERIMENTS.

Comparison between the Immunity produced by Ingestion and by Subcutaneous Inoculation.

Since the discovery of *B. dysenteriae* by Shiga, a practical method of prophylactic inoculation against dysentery has been the subject of many investigations. The high degree of toxicity of this organism has rendered the solution of this problem difficult, and many efforts have been made to reduce the toxicity of the vaccine without interfering with its power to confer immunity. Shiga conceived the idea of inoculating a mixture of killed bacilli and anti-serum. The introduction of sensitised vaccines on the principle of Besredka carried the idea a stage further, and experiments in this connection by Dopter (1909) and Broughton-Alcock (1914) may be here cited. The latter reported good results obtained with a vaccine, the toxicity of which had been reduced by treating the bacterial bodies with heated normal serum. Gibson (1917), Ruffer and Willmore (1918) amongst others have added to our knowledge in this field, and more recently the efficacy of lipo-vaccines has been investigated by Whitmore and Fennel (1918) and Olitsky (1918). In most of this work the titre of antibody has been accepted as a measure of the degree of immunity obtained, but evidence is accumulating that the two do not run a parallel course. From a comparative study of the degree of active immunity produced by different types of dysentery vaccines (heat killed, carbolised, treated with normal or with immune serum), J. D. Thomson (1916) concluded that the carbolised vaccine, whilst giving rise to the least reaction, was productive of the highest degree of immunity. It was decided, therefore, to compare the protection afforded by oral immunisation with that resulting from the subcutaneous inoculation of carbolised vaccines.

Preparation of carbolised vaccine.—This was prepared in the usual manner by suspending the 24-hour growth on agar of *B. dysenteriae* (Shiga) in 0·5 per cent. carbolic acid in saline. The suspension was counted and the doses required made up to 1·0 c.c. with saline as required.

Administration of vaccine per os.—In these experiments the vaccine was delivered into the stomach by means of a small rubber cannula to which was affixed a small filter funnel. In this experiment 24 rabbits of suitable size were taken and divided into 2 series of 12 each. Series 1, which received the vaccine *per os*, was further subdivided into two groups, A and B, which were treated as follows:

SERIES I.

Group A : Rabbits Nos: 27, 28, 29, 30, 31 and 32.

Each rabbit in this group received three doses by the oral route of a suspension of *B. dysenteriae* (Shiga), killed by heating to 60° C. The dose in each case was $\frac{1}{5}$ of a Roux bottle culture and the interval between the dose ten days.

Group B : Rabbits Nos. 33, 34, 35, 36, 37 and 38.

These rabbits were immunised by the oral route, receiving similar doses to those given to Group A, with, however, the addition of 8·0 c.c. of sterile ox bile with each dose of vaccine. One of these animals—No. 35—died three days after the first dose from pneumonia.

SERIES II.

This series consisted of 12 rabbits which were immunised by subcutaneous inoculation of a carbolised vaccine of dysentery bacilli (Shiga). Three doses were given, one of 50 million and two of 100 million, the interval between the doses being ten days. Rabbits Nos. 40 and 52 died five days after the first inoculation, post-mortem examination revealing some congestion of the right lung, with oedema and injection of the small intestine.

The surviving animals of these two series were tested for their immunity to living *B. dysenteriae* (Shiga) three weeks after the last immunising dose. The test doses employed were 1000 millions and 2000 millions, and were given intravenously. The results of this experiment are tabulated below:

TABLE IV.

No. of rabbit.	Immunisation.	Test dose.	Result.
27 .	3 doses ($\frac{1}{5}$ Roux bottle) per os	1000 million .	Died on the 5th day.
28 .	„	„	Died on the 5th day.
29 .	„	„	Survived.
33 .	„ + ox bile	„	Died on the 5th day.
34 .	„ „	„	Died on the 5th day.
30 .	3 doses ($\frac{1}{5}$ Roux bottle) per os	2000 million .	Survived.
31 .	„	„	Died on the 2nd day.
32 .	„	„	Died on the 5th day.

TABLE IV—*continued*.

No. of rabbit.	Immunisation.	Test dose.	Result.
36 .	3 doses ($\frac{1}{5}$ Roux bottle) + ox bile .	2000 million .	Died on the 2nd day.
37 .	„ „ .	„ .	Survived.
38 .	„ „ .	„ .	Paralysed—recovered.
39 .	Subcutaneous inoculation of carbolised vaccine, 3 doses (50, 100 and 100 million)	1000 million .	Survived.
41 .	Ditto	„ .	Survived.
42 .	„	„ .	Survived.
43 .	„	„ .	Survived.
44 .	„	„ .	Survived.
47 .	„	2000 million .	Survived.
48 .	„	„ .	Survived.
49 .	„	„ .	Survived.
50 .	„	„ .	Survived.
51 .	„	„ .	Died on the 5th day.
Body weight.			
54 .	Controls {	2000 gram. .	500 million . Survived.
57 .		1450 „ .	„ . Died on the 3rd day.
55 .		1860 „ .	1000 million . Died on the 2nd day.
58 .		1974 „ .	„ . Died on the 2nd day.
56 .		1700 „ .	2000 million . Died on the 2nd day.
59 .		2000 „ .	„ . Died on the 3rd day.

The surviving rabbits were observed over a period of three weeks. During the first two weeks there was some loss of weight, but by the end of the third week an increase in weight was recorded.

Post-mortem findings.—In the case of the control rabbits the mucous membrane of the small intestine was œdematous and injected. Nos. 55, 58 and 59 showed some hæmorrhages in the lungs. Those rabbits immunised *per os* which succumbed to the test-dose showed the same changes as the control animals though they were less acute. Rabbit No. 51, the only animal of Series II which died after receiving the test dose, showed rather severe changes. Hæmorrhages were present both in the lungs and large intestine.

DISCUSSION.

From the above experiment it will be seen that while the rabbits immunised *per os* had acquired a certain degree of resistance to the intravenous inoculation of *B. dysenteriae* (Shiga) (3 survivals out of 11 possibles), a much more solid immunity (9 survivals out of 10 possibles) had resulted from the subcutaneous inoculation of a carbolised vaccine. It is noteworthy also that the ingestion of bile did not seem to increase the efficacy of oral immunisation. Besredka drew attention to this point also, and states that the addition of bile was without result in the immunisation against *B. dysenteriae*. It might be pointed

out also, that in the first series the oral immunisation was carried out by feeding the rabbits with the killed bacilli, whereas in the second series the vaccine was introduced into the stomach by means of a cannula. In neither case did any solid immunity result.

CONCLUSIONS.

(1) It has been possible to produce a certain small degree of immunity in rabbits by the oral administration of *B. dysenteriae* (Shiga).

(2) The immunity so obtained is found to be far inferior to that produced by the subcutaneous inoculation of a carbolised vaccine administered in three doses of 50, 100 and 100 millions.

(3) The oral administration of large quantities of killed dysentery bacilli to rabbits is without effect on their general condition as judged by the body weight.

To many members of the Bacteriological Staff of this Institute I am indebted for helpful advice and criticism.

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FURTHER EXPERIMENTAL STUDIES ON IMMUNISATION AGAINST *B. DYSENTERIÆ* (SHIGA) AND ITS TOXINS.

S. KANAI, M.D. (Tokyo).

From the Bacteriological Department, Lister Institute, London.

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IN a previous communication (Kanai, 1921) experimental evidence has been brought forward to show that rabbits immunised by the subcutaneous administration of a carbolised vaccine of *B. dysenteriae* (Shiga) acquired a more solid immunity to living Shiga bacilli intravenously administered than those immunised *per os*. In the course of this work it was noted that those animals which succumbed to the test inoculation invariably showed some paralysis prior to death, with the constant post-mortem finding of hæmorrhages in the medulla and spinal cord, whereas any pathological change in the intestinal mucosa apart from œdema was of rare occurrence. This mode of reaction on the part of the rabbit to dysentery toxin is well known, and the difference in behaviour of this animal and the guinea-pig to the toxin of *B. dysenteriae* has led to the conclusion that there exist in reality two toxins. The one, especially toxic for the rabbit, gives rise to the paralytic symptoms, the other produces pyrexia and general marasmus and is more readily demonstrated in the guinea-pig. These two toxins have been variously referred to as "rabbit toxin" and "guinea-pig toxin," or "paretische Gift" and "marantische Gift" (Pfeiffer, 1908), and attempts to establish the nature of these toxins have been productive of much discussion. It would be impossible in the space of a short article to review the literature on this question, but suffice it to say that up till quite recently it remained undecided. The recent researches of Olitsky and Kligler (1920), however, would appear to offer a solution of this question in so far as the effect on the rabbit is concerned. These workers claim to have demonstrated unequivocally the presence of two types of dysentery toxin. One is soluble, a true exotoxin, is destroyed by heating at 80° C., and is responsible for the paralytic symptoms in the rabbit. It is essentially a neurotoxin and gives rise to those hæmorrhages in the medulla and cord to which reference has already been made. The other type they classify with the so-called endotoxins. It is more heat-stable than the neurotoxins, withstands a temperature of 80° C., and produces the hæmorrhages in the intestinal mucosa which determine the characteristic lesion of dysentery.

Besredka (1919) claims, in his recent publication on immunisation by the oral route, that the immunity produced in animals by this method to *B. dysenteriae* (Shiga) is a local immunity. The cells of the intestinal mucosa have acquired a new character; they are immune to dysentery toxin. Was it

possible, then, that those rabbits which in our last study had been immunised *per os* and which were unable to withstand the test-dose of living bacilli, succumbed to the neurotoxin (exotoxin) alone, and that they were immune to the so-called endotoxin, which, according to Olitsky, produces the hæmorrhagic lesions in the intestinal wall? It may be recalled that these animals showed lesions in the central nervous system only. It was decided to test the correctness of this hypothesis.

OUTLINE OF THE PROPOSED EXPERIMENTS.

It is well known to all who have studied the toxicity of dysentery bacilli that strains of the same organism differ markedly in their power to produce intestinal lesions in the rabbit. It was proposed first of all, therefore, to see if a strain of *B. dysenteriae* (Shiga) could be found which produced chiefly intestinal lesions. It was thought that with such a strain it would be possible to test the local immunity produced by the oral administration of the vaccine.

TABLE I.

Rabbit.	No. of strain.	Symptoms.	Dysentery bacilli recovered from—	Post-mortem findings.
No. 60. 1400 gr.	151.	Paralysed on 1st day, died 28 hours after injection.	Gall-bladder, spleen and kidney.	<i>Lung</i> : Hæmorrhages. <i>Small intestine</i> : Injection of mucous membrane and leucocytic infiltrations of tunica propria.
No. 61. 1870 gr.	152.	Found dead 19 hours after injection.	Spleen.	No hæmorrhages in lung, otherwise changes similar to those in No. 60 were observed.
No. 62 1630 gr.	208.	Paralysed on 3rd day. Died on the 5th day. No diarrhœa.	—	<i>Small intestine</i> : Hyperæmia and œdema. <i>Appendix</i> shows similar changes. <i>Large intestine</i> appears normal.
No. 63. 1580 gr.	753.	Paralysed on 1st day, died on the 2nd day, no diarrhœa.	Gall-bladder, appendix and lung.	<i>Lung</i> : Hæmorrhages. <i>Liver</i> : Congested. <i>Small intestine and duodenum</i> : Some œdema and hyperæmia.
No. 64. 1330 gr.	752.	Found dead next morning.	Gall-bladder, spleen and kidney.	Same as in Rabbit 63.
No. 65. 1840 gr.	754.	Paralysed on 2nd day, died on 3rd day.	Gall-bladder and spleen.	No hæmorrhages in lung or noteworthy changes in gut-wall.
No. 78. 1500 gr.	Besredkas' strain, $\frac{1}{2}$ of an agar slope.	No paralysis and no diarrhœa. Rabbit found dead on the 2nd day.	Gall-bladder and spleen.	<i>Lung</i> : Small hæmorrhages. <i>Liver and kidney</i> : Congested. <i>Small intestine</i> : Œdema and injection of submucosa. <i>Large intestine</i> : Œdema and injection with some hæmorrhages in submucosa and tunica propria.
No. 77. 1380 gr.	The above strain, $\frac{1}{5}$ of an agar slope (weak growth).	No symptoms; survived.		

Further, the preparation of dysentery exo- and endotoxin, on the lines of Olitsky's work, was envisaged, with the object of testing the relative immunity of rabbits, immunised subcutaneously and orally, to these two toxins.

COMPARISON OF THE TOXICITY OF VARIOUS STRAINS OF *B. DYSENTERIÆ* (SHIGA).

Seven strains of *B. dysenteriae* (Shiga) were taken and injected intravenously into rabbits in a dose of one-fifth of a 24-hour agar slope culture. This quantity of bacilli was suspended in 1.0 c.c. of normal saline. Those rabbits which died as the result of the inoculation of the living bacilli were examined for the presence of hæmorrhagic lesions—particularly in the intestine—while the gall-bladder, heart-blood, spleen, kidney, lung and liver were submitted to cultural investigation for the presence of dysentery bacilli. The results of this experiment are given in Table I.

Of the seven strains employed in this experiment Besredka's alone appears capable of producing hæmorrhages in the intestine, but even with this strain they were of a trivial character. It was decided, therefore, to proceed with the preparation of exo- and endotoxin with which to test the local immunity of the immunised rabbits.

PREPARATION AND CHARACTERS OF DYSENTERY TOXINS.

Exotoxin.

The growths from fifteen 24-hour agar slopes (strain No. 151) were suspended in 10.0 c.c. normal saline and immediately filtered through a Berkefeld filter-candle (N). The filtrate was tested on rabbits by intravenous inoculation and the results obtained are recorded below (Table II).

TABLE II.

Rabbit.	Body-weight.	Dose injected.	Symptoms.	Post-mortem findings.
No. 88.	1400 grs.	5.0 c.c.	Paralysis of fore-legs 1st day after inoculation. No diarrhoea. Found dead on 2nd day.	<i>Lungs, liver and kidneys</i> are congested. <i>Spleen</i> congested, Malpighian bodies swollen, and show some small hæmorrhages. <i>Large intestine</i> : Œdema and injection of submucosa and tunica propria, with some extravasation of red cells in the œdematous portions. <i>Small intestine</i> : Injection and œdema of submucosa. <i>Medulla and spinal cord</i> . Pia œdematous and vessels dilated. Some hæmorrhages in the grey substance and chromatolysis of nerve-cells.
No. 76.	2330 grs.	3.0 c.c.	Dyspnœa 2 hours after injection lasting 2 hours. Hind legs paralysed next morning. Died 28 hours after inoculation.	Similar to those observed in No. 88.
No. 77.	2230 grs.	1.0 c.c.	Hind legs paralysed on 2nd day. Died 3 days after injection.	—

It will be seen from this experiment that when a 24-hour agar culture of *B. dysenteriae* (Shiga) is suspended in normal saline and immediately freed from the bacillary bodies by filtration, the filtrate is highly toxic for rabbits, producing the same symptoms and post-mortem changes as the whole bacilli.

Resistance of "exo-toxin" to heat.—Exotoxin, prepared as in the previous experiment, was submitted to temperatures of 60° C. and 80° C. for one hour and inoculated intravenously into rabbits in doses of 1.0 c.c., with the following results :

TABLE III.

Rabbit.	Body-weight.	Toxin.	Results.
No. 83.	1320 grs.	1.0 c.c. "exotoxin" heated at 80° C.	No symptoms. Survived.
No. 84	1100 grs.	1.0 c.c. "exotoxin" heated at 60° C.	Paralysis 1st day. Found dead on morning of 2nd day.

Post-mortem findings in Rabbit No. 84 :—*Lungs* : Congestion and hæmorrhages. *Spleen and kidney* : Congested. *Liver* : Congested—shows evidence of infection with coccidia. *Small intestine* : Injected. Polynuclear infiltration of submucosa, with some hæmorrhages. *Large intestine, appendix* : Normal except for presence of coccidia.

It is seen, then, from this experiment that the readily soluble portion of the dysentery toxins (exotoxin) is rendered non-toxic for the rabbit by heating it at 80° C. for one hour, whereas a temperature of 60° C. leaves it capable of producing both lesions in the central nervous system and hæmorrhage in the intestinal mucosa.

Minimum lethal dose of "exotoxin."—An attempt to determine the M.L.D. of this exotoxin gave inconclusive results. Three rabbits were inoculated intravenously with 0.1 c.c., 0.5 c.c. and 1 c.c. respectively. The two which received 0.1 c.c. and 1.0 c.c. survived, having shown no symptoms whatever, whereas the third with 0.5 c.c. became paralysed on the first day, recovering on the fourth day after inoculation.

Endotoxin.

It was held by Klein (1907) and Heller (1909) that the toxin of *B. dysenteriae* (Shiga) was of the nature of an endotoxin, but, as already stated, the experiments of Olitsky and Kligler would appear to demonstrate the existence of two dysentery toxins, the one soluble, destroyed at 80° C.—neurotoxin—the other remaining attached to the bacillary bodies, resisting a temperature of 80° C. and giving rise to the hæmorrhagic lesions in the intestine. The following experiments were undertaken to determine the toxicity of dysentery bacilli after removal of the soluble toxin by washing in normal saline. The strains employed were No. 151 and Besredka's.

Toxicity of bacilli washed in saline five times.—The growth obtained from fifteen 24-hour agar slopes was suspended in 5.0 c.c. normal saline and

centrifuged to throw down the bacilli. The clear supernatant fluid was pipetted off and an additional 5.0 c.c. saline added, in which the deposit was re-suspended. This was repeated five times. The final suspension was heated at 60° C. for one hour, centrifuged again, and the toxicity of the supernatant fluid and the bacillary deposit tested on rabbits (Table IV).

TABLE IV.

Rabbit.	Body-weight.	Inoculum.	Result.
No. 92.	1500 grs.	500 × 10 ⁶ washed bacilli.	Survived.
No. 90.	1520 grs.	1000 × 10 ⁶ washed bacilli.	Hind legs paralysed 2nd day. Killed 3rd day for examination.
No. 91.	1300 grs.	2000 × 10 ⁶ washed bacilli.	Forelegs paralysed 1st day. Died 2nd day.
No. 93.	1550 grs.	3.0 c.c. supernatant.	Died 2nd day.

Post-mortem findings in rabbit No. 90:—*Lungs*: Congested. *Spleen*: Hæmorrhages in pulp, and some necrosis of the cells in the germ centres. *Liver*: Congested. *Small intestine*: Vessels of submucosa dilated and some œdema of the tunica propria. *Large intestine*: Œdema. Some small hæmorrhages in tunica propria. *Spinal cord*: Numerous small hæmorrhages in grey matter and chromatolysis of nerve-cells.

Rabbit No. 93 showed very similar changes.

Effect of a temperature of 80° C. on endotoxin.—The same bacterial emulsion, washed five times (as used in the last experiment), was heated at 80° C. for one hour and three rabbits inoculated intravenously with varying doses. The result of this experiment is given in Table V.

TABLE V.

Rabbit.	Body-weight.	Dose inoculated.	Result.
No. 94.	1210 grs.	2000 × 10 ⁶	Survived. No symptoms.
No. 95.	1300 grs.	1000 × 10 ⁶	„ „
No. 96.	1880 grs.	4000 × 10 ⁶	„ „

Rabbit No. 96 was killed and examined, the post-mortem findings being as follows:—*Lungs*: Congested. *Spleen*: Pulp congested and shows hæmorrhages. *Kidney*: Hæmorrhages in cortex. *Small intestine*: Injection of vessels of villi and œdema of tunica propria. *Large intestine*: Injection of vessels of tunica propria. Some small hæmorrhages in wall of appendix. *Spinal cord and medulla*: No change.

This experiment was repeated, but a suspension of unwashed bacilli was employed in place of bacilli washed five times in normal saline. The suspension was heated for one hour at 80° C. as before.

TABLE VI.

Rabbit.	Body-weight.	Dose inoculated.	Result.
No. 132.	1920 grs.	5000 \times 10 ⁶ .	No symptoms.
No. 131.	1900 grs.	10,000 \times 10 ⁶ .	„
No. 135.	2010 grs.	20,000 \times 10 ⁶ .	Diarrhœa. No paralysis. Found dead 1st day after inoculation.
No. 136.	2040 grs.	40,000 \times 10 ⁶ .	Weak 1st day after inoculation. Recovered.

Post-mortem findings in Rabbit 135:—*Lungs*: Hæmorrhages in right lobe. *Spleen*: Severe congestion of pulp and small hæmorrhages in folliculi. *Liver*: Hæmorrhages. Necrosis of parenchyma with leucocytic infiltration of necrotic areas. *Kidneys*: Congestive swelling of convoluted tubules. *Small intestine*: Hyperæmia of tips of villi. *Stomach*: Hæmorrhages in submucosa. *Large intestine*: Œdema and hæmorrhages in tunica propria (heavily infected with coccidia). *Spinal cord*: Small capillary hæmorrhages in grey matter of cervical portion and in medulla.

From the above experiments it would appear that the readily soluble portion of the toxin of dysentery bacilli, the so-called exotoxin, produces changes chiefly in the central nervous system. These are of the nature of capillary hæmorrhages in the grey matter of the cord and medulla and chromatolysis of the nerve-cells. The action of this exotoxin is not confined to the nervous system, however, as a general action on the circulatory system is evinced by capillary hæmorrhages throughout the body. Thus hæmorrhages were noted in the lungs, liver, kidney, spleen, and especially in the submucosa of intestine, which invariably showed some œdema accompanied as a rule by extravasation of red cells. Heating for one hour at 60° C. did not reduce the toxicity of this soluble portion of the dysentery toxin.

From the experiments carried out with the washed bacilli it is evident that a large proportion of the toxin still remains closely united with the bacterial bodies, even after they have been washed five times and extracted with normal saline for one hour at a temperature of 60° C. Bacilli so treated were still highly toxic for rabbits, producing much the same changes as those arising from the inoculation of the soluble portion of the dysentery toxin. When these washed and extracted bacilli are submitted to a temperature of 80° C. for one hour their toxicity for the rabbit is greatly reduced. Of the three rabbits inoculated with bacilli so treated none died or showed any symptoms whatever. The findings in one of these three rabbits, which was killed and examined, showed complete absence of change in the central nervous system, though there were hæmorrhages in the various viscera. These experiments were carried out with strain No. 151, and it must be admitted that the attempts to prepare an exo- and endotoxin from it were not attended with any great success, though bacilli when heated at 80° C. seemed to have lost to a certain extent their neurotoxin and were yet capable of giving rise to hæmorrhages, particularly in the intestinal submucosa. The following experiment was therefore carried out with Besredka's strain, to see whether it would be possible to achieve with another strain of *B. dysenteria* (Shiga) what had

proved unsuccessful with strain 151, viz. the separation of exotoxin from endotoxin. This experiment is summarised in Table VII.

TABLE VII.

Rabbit.	Material inoculated.	Result.
No. 123.	Fifth washing of the growth from ten agar slopes. (Bacilli washed each time with 5.0 c.c. saline, fifth washing filtered through a Berkefeld filter N.)	Paralysis of forelegs on 1st day. Died 2 days after inoculation.
No. 124.	The washed bacilli from above, suspended in saline, heated at 60°C. for one hour and centrifuged. Bacilli so obtained inoculated intravenously in a dose of 2000×10^6 .	Paralysed and died the 1st day after inoculation.
No. 126.	The tenth washing of five agar slope cultures. (Bacilli washed each time with 5.0 c.c. saline, the tenth washing filtered through a Berkefeld filter N.)	Survived. No symptoms.
No. 127.	The bacilli after tenth washing, heated for one hour at 60°C., washed three times with normal saline (5.0 c.c. saline on each occasion). Bacilli so treated inoculated intravenously in a dose of 3000×10^6 .	No paralysis. No diarrhoea. Died suddenly on 3rd day.
No. 125.	Bacilli as prepared for inoculation of Rabbit No. 127 heated for one hour at 80°C. and inoculated intravenously in a dose of 4000×10^6 .	Survived. No symptoms.
No. 128.	Twenty-four hour growth on agar suspended in normal saline, heated for one hour at 80°C. and inoculated intravenously in a dose of $10,000 \times 10^6$.	No diarrhoea. No paralysis. Died 1st day after inoculation.

The post-mortem examination of those rabbits which succumbed revealed precisely the same changes as those produced by similar preparations made with strain No. 151.

It will be seen, then, from the above experiment that the attempt to separate endo- and exotoxin from Besredka's strain of *B. dysenteriae* (Shiga) had not been attended with any more success than in the case of strain No. 151. The experiments, however, demonstrate the fact that when a 24-hour culture of *B. dysenteriae* (Shiga) is suspended in normal saline and the bacilli then removed by centrifugalisation and filtration, the filtrate is highly toxic for the rabbit, *giving rise to similar changes to those produced by the whole bacilli*. Both these two fractions, the supernatant fluid and the washed bacilli, appear to contain the two toxins, the neurotoxin and the hæmorrhagic toxin, but by heating the washed bacilli for one hour at 80°C. their power of producing lesions in the central nervous system is destroyed. They are still capable, however, of giving rise to hæmorrhages in the various viscera when inoculated intravenously, these changes occurring constantly in the submucosa of the intestine. A temperature of 60°C. is without effect on the toxicity of these fractions of dysentery toxin.

RESISTANCE OF IMMUNISED RABBITS TO TOXIN FRACTIONS.

It now remained to test the immunity of rabbits, immunised orally and subcutaneously, to these two fractions of dysentery toxin. Before doing this,

however, it was decided to see if any pathological changes occurred in rabbits as the result of the administration of large doses of dysentery vaccine *per os*. Five rabbits were taken and to each was given one-fifth of a Roux-bottle culture of *B. dysenteriae* (Shiga), killed by heating for one hour at 60° C. These rabbits were killed at varying intervals after the ingestion of vaccine and the post-mortem finding are given below (Table VIII).

TABLE VIII.

Rabbit.	Time of killing.	Post-mortem findings.
No. 97 (1325 grs.).	4 hrs. after ingestion.	Some congestion of appendix, otherwise normal.
No. 99 (1750 grs.).	24 hrs. „ „	Congestion of appendix, and tips of villi in small intestine. Large intestine showed presence of coccidia.
No. 100 (1850 grs.).	48 hrs. „ „	<i>Spleen</i> enlarged, congested. Shows some nodular lesions. <i>Liver</i> shows nodular lesions. <i>Kidney</i> hæmorrhages.
No. 101 (1660 grs.).	96 hrs. „ „	<i>Small intestine</i> : Congestion of submucosa with some petechial hæmorrhages. <i>Appendix</i> : Congestion and eosinophile infiltration—coccidia. <i>Kidney</i> : Congestion. <i>Liver</i> : Hæmorrhages. <i>Spinal cord</i> : Capillary hæmorrhages in cervical portion.
No. 98 (1550 grs.).	2 weeks „ „	<i>Small intestine</i> : Congestion. <i>Large intestine</i> : One or two hæmorrhages. <i>Coccidia</i> . <i>Appendix</i> : Congestion and œdema of tunica propria. <i>Kidneys</i> : Hæmorrhages, with accompanying degenerative changes in parenchyma. <i>Spinal cord and medulla</i> : Congestion and capillary hæmorrhage.

Unfortunately, four out of the five rabbits were infected with coccidiosis, which detracted to a great extent from the value of this control experiment, as attention has frequently been drawn to the production of hæmorrhages in rabbits by this parasite. However, the experiment is not without interest. Undoubtedly some of the hæmorrhages were due to coccidiosis, but in rabbits Nos. 101 and 98 these occurred in kidney and central nervous system, where no parasites could be found. It seems legitimate to conclude, therefore, that these latter hæmorrhages were the result of the ingestion of dysentery vaccine, the dysentery toxin having passed through the intestinal wall into the general circulation.

Six rabbits were immunised by the oral administration of a Shiga vaccine (strain No. 151, killed at 60° C.), the dose employed being one-fifth of a Roux-bottle culture, and three doses being given at 10 days' interval. Another series of six rabbits received 3 doses of a carbolised vaccine of the same strain inoculated subcutaneously, the interval between the injections being again 10 days. The doses employed were: 1st dose, 50×10^6 ; 2nd dose, 100×10^6 ; 3rd dose, 200×10^6 .

Ten days after the last immunising dose four rabbits in each series were given varying doses of the soluble dysentery toxin intravenously, whilst the remaining two animals in each series received intravenous doses of washed dysentery bacilli which had been submitted to a temperature of 80° C.

The dose of washed bacilli (heated to 80° C.) was fixed at $20,000 \times 10^6$ (from result of experiment detailed in Table VI), but as this proved insufficient to kill the control, Table IX (annexed) gives no information as to immunity to toxic substances present in the washed and heated bacilli.

TABLE IX.

A.—Rabbits Immunised by Subcutaneous Route.

Rabbit.	Weight.	Test-dose.	Result.
No. 102.	1350 grs.	0.7 c.c. "soluble" toxin intravenously.	Paralysed on 2nd day. Recovered on 4th day. No further symptoms. Killed and examined. <i>Lung</i> : Congestion and hæmorrhages. <i>Spleen</i> : Severe congestion and hæmorrhages in pulp. <i>Liver</i> : Small hæmorrhages. <i>Small intestine</i> : Congestion and œdema of submucosa. <i>Spinal cord and medulla</i> : Hæmorrhages in grey substance.
No. 193.	1650 grs.	0.7 c.c. "soluble" toxin intravenously.	Paralysed on 2nd day. Died on 4th day. <i>Lung</i> : Congestion and serous exudate in alveoli. <i>Spleen</i> : Malpighian bodies show hæmorrhages and œdema of germ centres. <i>Small intestine</i> : Injection of submucosa. <i>Large intestine</i> : Injection of submucosa. <i>Appendix</i> : Eosinophile infiltrate. Coccidiosis. <i>Spinal cord</i> : Vessels of grey substance injected—some hæmorrhages. Nerve-cells show chromatolysis and some vacuolisation. <i>Medulla</i> : Injection of vessels and hæmorrhages in grey substance.
No. 104.	1150 grs.	1.4 c.c. "soluble" toxin intravenously.	Hind legs became paralysed 20 hours after inoculation. Died soon after. <i>Lung</i> : Severe congestion and hæmorrhage. <i>Spleen</i> : Congestion and hæmorrhage. <i>Liver</i> : Congestion and hæmorrhage, some fibrosis and infiltration with eosinophile leucocytes. <i>Small intestine</i> : Submucosa shows injection of vessels, with small hæmorrhages. <i>Large intestine</i> : Tunica propria shows injection of vessels, œdema, eosinophilic infiltrate and many small hæmorrhages. <i>Spinal cord, medulla</i> : Hæmorrhages and degenerative changes in nerve-cells.
No. 105.	1600 grs.	Ditto.	No symptoms. Survived. Killed and examined. <i>Lung, liver and spleen</i> : Congestion. <i>Small intestine</i> : Œdema. Injection of vessels and hæmorrhages in tunica propria. <i>Large intestine</i> : Injection and œdema of submucosa. <i>Spinal cord and medulla</i> : Nil. Rabbit infected with coccidia.
No. 106.	2150 grs.	$20,000 \times 10^6$ washed bacilli killed by heating at 80° C. for one hour.	No symptoms. Survived. Killed and examined. <i>Lungs</i> : Nil. <i>Spleen</i> : Enlarged. Swelling of cells in germ centres. <i>Small intestine</i> : Small macroscopic hæmorrhages. <i>Large intestine</i> : Many small hæmorrhages and œdema of tunica propria. <i>Spinal cord</i> : Injection of vessels in grey matter. <i>Medulla</i> : Nil.
No. 107.	2000 grs.	Ditto.	No diarrhœa. No paralysis observed. Found dead on morning of 3rd day. <i>Lung</i> : Œdema and fibrinous exudate in alveoli. <i>Liver and kidney</i> : Congested. <i>Small intestine</i> : Injection of submucosa. <i>Large intestine</i> : Nil. <i>Spinal cord</i> : Large hæmorrhages in grey matter of cervical and lumbar regions. Nerve-cells show degenerative change. <i>Medulla</i> : Injection of grey matter.

TABLE IX—*continued*.B.—*Rabbits Immunised per os*.

Rabbit.	Weight.	Test-dose.	Result.
No. 111.	1640 grs.	0.7 c.c. "soluble" toxin intravenously.	No symptoms. Survived.
No. 112.	1440 grs.	Ditto.	Paralysis of hind legs on 2nd day. Died 3rd day. <i>Lung</i> : Congestion and hæmorrhage. <i>Liver</i> : Coccidiosis. <i>Small intestine</i> : Œdema, injection of submucosa. <i>Large intestine</i> : Slight œdema. <i>Spinal cord and medulla</i> : Degenerative change in nerve-cells, hæmorrhage.
No. 113.	1900 grs.	1.4 c.c. "soluble" toxin intravenously.	Paralysed on 2nd day. Died 3rd day. <i>Lung</i> : Congestion and hæmorrhages. <i>Spleen, liver and kidney</i> : Congestion. <i>Small intestine</i> : Injected. Œdema. <i>Large intestine</i> : Injection of submucosa. Œdema. <i>Appendix</i> : Injected. <i>Spinal cord</i> : Hæmorrhages in grey substance and degenerative change in nerve-cells. <i>Medulla</i> : As spinal cord.
No. 114.	1300 grs.	Ditto.	No symptoms. Survived. Killed and examined. <i>Lung, Liver and Kidney</i> : Nil. <i>Spleen</i> : Congestion. Cells of germ centres swollen. <i>Small intestine</i> : Injection and œdema of tunica propria, some small capillary hæmorrhages. <i>Spinal cord and medulla</i> : Nil.
No. 115.	1760 grs.	20,000 \times 10 ⁶ washed bacilli killed at 80° C. injected intravenously.	No symptoms. Survived. Killed and examined. <i>Lung</i> : Nil. <i>Spleen</i> : Severe congestion of pulp. <i>Small intestine</i> : Injection of tunica propria. <i>Spinal cord and medulla</i> : Nil.
No. 116.	1760 grs.	Ditto.	No symptoms. Survived. Killed and examined. Similar changes to those found in Rabbit 115.

C.—*Control Rabbits*.

No. 129.	1060 grs.	0.7 c.c. "soluble" toxin intravenously.	Found dead on morning of 2nd day. <i>Lung</i> : Congestion and hæmorrhage. <i>Spleen</i> : Severe congestion. <i>Liver</i> : Congestion and hæmorrhage. <i>Pancreas</i> : Hæmorrhages. <i>Kidney</i> : Congestion. <i>Small intestine</i> : Injection and œdema of submucosa, with eosinophile infiltration. <i>Appendix</i> : Eosinophile infiltrate. <i>medulla</i> : Hæmorrhages. <i>Spinal cord</i> : Injection of grey matter.
No. 130.	1200 grs.	1.4 c.c. "soluble" toxin intravenously.	Found dead on morning of 2nd day. Post-mortem findings as in Rabbit 129.
No. 137.	1900 grs.	20,000 \times 10 ⁶ washed bacilli killed at 80° C. injected intravenously.	No symptoms. Survived. Killed and examined. <i>Lung, liver, kidney</i> : Nil. <i>Spleen</i> : Enlarged. <i>Small intestine</i> : Injected. <i>Large intestine</i> : Nil. <i>Spinal cord and medulla</i> : Nil.

The titre of agglutinins for *B. dysenteriae* (Shiga) in the serum of each of these rabbits was estimated ten days after the third immunising dose of vaccine. The suspension employed was made from a 24-hour agar culture and contained 2000 \times 10⁶ bacilli per c.c. Results were read after 4 hours at 37° C. and 24 hours at room temperature.

TABLE X.

A.—Rabbits injected subcutaneously with carbolised vaccine.

Rabbit	No. 102	No. 103	No. 104	No. 105	No. 106	No. 107
Agglutinin titre for <i>B. dysenteriae</i> (Shiga)	1/80	1/160	1/160	1/320	1/320	1/320

B.—Rabbits immunised *per os*.

Rabbit	No. 111	No. 112	No. 113	No. 114	No. 115	No. 116
Agglutinin titre for <i>B. dysenteriae</i> (Shiga)	1/10	<i>Nil</i>	<i>Nil</i>	1/40	1/40	<i>Nil</i>

From the experiment given in Table IX it will be seen that a certain degree of immunity can be produced in rabbits to the readily soluble fraction of dysentery toxin (so-called exotoxin) by both methods of immunisation. As to the degree of immunity in the two series A. and B. there is little to choose. Certainly those rabbits which had received subcutaneous inoculations of a carbolised vaccine showed more extensive pathological changes in the intestine and hæmorrhages were more numerous, which fact might, at first sight, be taken as evidence of some local immunity in the intestinal wall produced by the ingestion of the heat-killed vaccine. A closer inspection of the evidence, however, reveals the fact that the rabbits in question, Nos. 104, 105 and 106, were all infected with coccidia, so that it was impossible to consider these hæmorrhages as necessarily due to the test dose of toxin. It was therefore impossible to demonstrate conclusively any local intestinal immunity. Those rabbits which died in both series A. and B. succumbed to the nerve toxin. The data given in Table X show that the subcutaneous administration of vaccine is productive of a higher antibody titre than the oral immunisation.

As the experiment above detailed did not yield conclusive data of comparative value, it was decided to repeat in the hope that a definite conclusion might be arrived at. Two series of rabbits, six animals in each series, were immunised as before, one lot by subcutaneous inoculation of a carbolised vaccine, the other by the oral administration of heat-killed bacilli. In this case total leucocyte counts were made daily on each rabbit, both during the immunisation and after the administration of the test dose of toxin, and the temperature of each animal was similarly recorded. The object of this was to obtain some idea of the influence of these two methods of immunisation on the rabbits, and also to obtain further information as to the comparative effect of the test dose of toxin on the animals of these two series.

It has been pointed out by Lüdke (1911) amongst others that a leucocytosis occurs after the injection of dysentery toxin. Space would not permit of the inclusion in this paper of these daily observations on the leucocytic count and body temperature. Generally speaking there was an increase in the total white count and a rise in temperature of 1°–3° C. following each immunising dose. These changes were more severe in those rabbits receiving the sub-

cutaneous inoculations; apparently the ingestion of the vaccine is productive of less general reaction. The observations made four hours after the inoculation of the test dose of soluble toxin intravenously showed a rise in temperature of $2-3^{\circ}\text{C}$. accompanied generally with a fall in the total leucocyte count. This leucopenia occurred in all six of the rabbits immunised *per os*, whereas two of the six animals which had been inoculated subcutaneously showed no fall in the leucocyte count, and in two others only a moderate degree of leucopenia resulted.

The figures bearing on this point are here annexed:

Orally Immunised Series: Total Leucocytes.

Before test dose intravenously	11,567	11,898	10,245	9,915	9,135
Four hours afterwards	1,718	5,783	2,223	4,296	1,718

Series immunised by the subcutaneous method.

Before test dose intravenously	9,915	8,758	11,567	9,414	8,265
Four hours afterwards	1,720	5,453	11,563	5,783	18,930

TABLE XI.

Series A.—Rabbits immunised *per os*. (Three doses of $\frac{1}{3}$ of a Roux bottle culture.)

Rabbit.	Test-dose.	Symptoms.	Result.
No. 138.	2.0 c.c. "soluble" dysentery toxin.	Diarrhœa. Paralysis of fore and hind legs.	Died, 2nd day.
No. 139.	Ditto.	No symptoms.	Survived.
No. 140.	"	Diarrhœa.	Died, 2nd day.
No. 141.	"	Diarrhœa. Paralysis of fore and hind legs.	Died, 4th day.
No. 142.	"	Diarrhœa. Incontinence of urine. Paralysis.	Died, 3rd day.
No. 143.	"	Diarrhœa. Paralysis of fore and hind legs. Incontinence of urine.	Died, 4th day.

Series B.—Rabbits immunised by subcutaneous inoculation. (Three doses 50, 100 and 100×10^6 .)

No. 144.	2.0 c.c. "soluble" dysentery toxin.	Paralysis of hind legs. No diarrhœa. Incontinence of urine.	Died, 4th day.
No. 145.	Ditto.	Paralysis of fore and hind legs. No diarrhœa. Recovered 7th day.	Survived.
No. 146.	"	Paralysis of fore and hind legs. Slight diarrhœa on 3rd day.	Survived.
No. 147.	"	Paralysis of hind legs. Diarrhœa.	Died, 3rd day.
No. 148.	"	Paralysis of hind legs. Diarrhœa.	Died, 4th day.
No. 149.	"	No symptoms.	Survived.

Five control animals were inoculated with varying doses of the soluble toxin with the following results:

Rabbit.	Weight.	Dose of toxin.	Result.
No. 155.	1350 grs.	0.1 c.c.	No symptoms. Survived.
No. 154.	1400 grs.	0.5 c.c.	Paralysed. Died 2nd day.
No. 153.	1500 grs.	1.0 c.c.	No symptoms. Survived.
No. 156.	1390 grs.	2.0 c.c.	Died, 24 hours after inoculation.
No. 157.	1440 grs.	2.0 c.c.	Paralysis of hind quarters and diarrhoea for two days. Recovered.

In Table XI the results of this experiment are detailed, the intravenous test dose of the soluble toxin being fixed at 2 c.c. Without reference to the unimmunised controls, which at the same time received doses varying from 0.1 c.c. to 2 c.c. (*bis*), the results would indicate a considerable superiority of subcutaneous over oral immunisation, there being in the one series three survivals out of six, and in the other, one only. If, however, recovery without symptoms be taken as the criterion of complete immunity there would appear to be only one such in each series. As had happened in a previous experiment when attempt was made to determine the minimum lethal dose of the soluble toxin, aberrant results occurred with the control animals of this experiment also, and probably, on the whole, it would be safest to conclude from the evidence available that solid active immunity to the soluble dysentery toxin is difficult and capricious by whatever method it is sought. No clear-cut evidence was obtained of an immunity similar to that which was realised in previous work when the test dose consisted of living dysentery bacilli.

The action of this soluble dysentery toxin intravenously administered has some points of resemblance with that of the so-called "shock" toxins, against which it is difficult in small animals to secure protection by active immunisation with killed vaccines, however administered.

ANTI-BODY RESPONSE TO ORAL IMMUNISATION.

The anti-body response to the ingestion of a dysentery vaccine was also investigated, the agglutination titre for *B. dysenteriae* (Shiga) of the serum of each rabbit of Series A. being determined one week after each dose of vaccine. The sera were collected and kept in the cold room and all tested together with the same suspension, which was made from a 24-hour agar culture and contained 2000×10^6 bacilli per c.c. The results are recorded in Table XII.

From Table XII it will be seen that the agglutination titre for *B. dysenteriae* (Shiga) rises appreciably with successive ingestions of bacilli. This is of interest, because Besredka's findings were entirely opposed to these. In his experiments he noted the appearance of agglutinins in the serum following the first ingestion of bacilli, after which they gradually disappeared, *the subsequent immunising doses of the vaccine giving rise to no anti-body response*. He

cites this observation in support of his contention that oral immunisation has produced an increased *local* resistance in the intestinal mucosa.

TABLE XII.

Time of observation.	Rabbit—				
	No. 144.	No. 145.	No. 146.	No. 147.	No. 148.
One week after 1st dose.	—	1/80	1/10	1/20	1/20
One week after 2nd dose.	1/10	1/80	1/20	1/80	1/20
One week after 3rd dose.	1/40	1/320	1/40	1/80	1/160

DISCUSSION.

The main object of these experiments, viz. the determination of the degree of local immunity in the intestinal mucosa produced by the ingestion of dysentery bacilli, was not achieved. The failure arose from two causes. First of all, no strain of *B. dysenteriae* (Shiga) was found which, when inoculated into rabbits, gave rise to intestinal lesions to the exclusion of other pathological changes, and secondly, all attempts to separate endotoxin from exotoxin, on the lines of Olitsky and Kligler's work, proved fruitless. Certainly a portion of the toxin is readily dissolved out of the bodies of the dysentery bacilli. It was only necessary to suspend an agar culture in normal saline and to remove the bacilli by centrifugalisation to obtain a highly toxic supernatant fluid. This soluble fraction, however, produced the same lesions in the rabbits as the whole bacilli—changes not wholly confined to the central nervous system—and when heated for one hour at 80° C. all toxicity was lost. It must be admitted that the bacilli, even after washing ten times, still retained some of their toxicity. But when bacilli so treated were submitted to a temperature of 80° C. for one hour their toxicity was practically destroyed. Out of six rabbits inoculated with washed and heated bacilli, only one died—the one which received $20,000 \times 10^6$ bacilli—and though this animal showed hæmorrhages in the intestinal mucosa and other viscera, the presence of a heavy coccidiosis infection rendered it doubtful whether these lesions were to be placed to the account of the dysentery toxin or not.

CONCLUSIONS.

(1) The toxin of *B. dysenteriae* (Shiga) affects principally the central nervous system (medulla and spinal cord) in rabbits. At the same time it acts upon the capillary circulation generally with the production of congestion and hæmorrhage in the various viscera.

(2) Exposure to a temperature of 80° C. for one hour markedly reduces its toxicity, especially for the central nervous system.

(3) It has been impossible to effect a separation between exotoxin and so-called endotoxin on the lines of Olitsky and Kligler.

(4) Though there is some indication in the foregoing experiments that oral immunisation produces a local immunity in the intestinal wall, this point remains undecided.

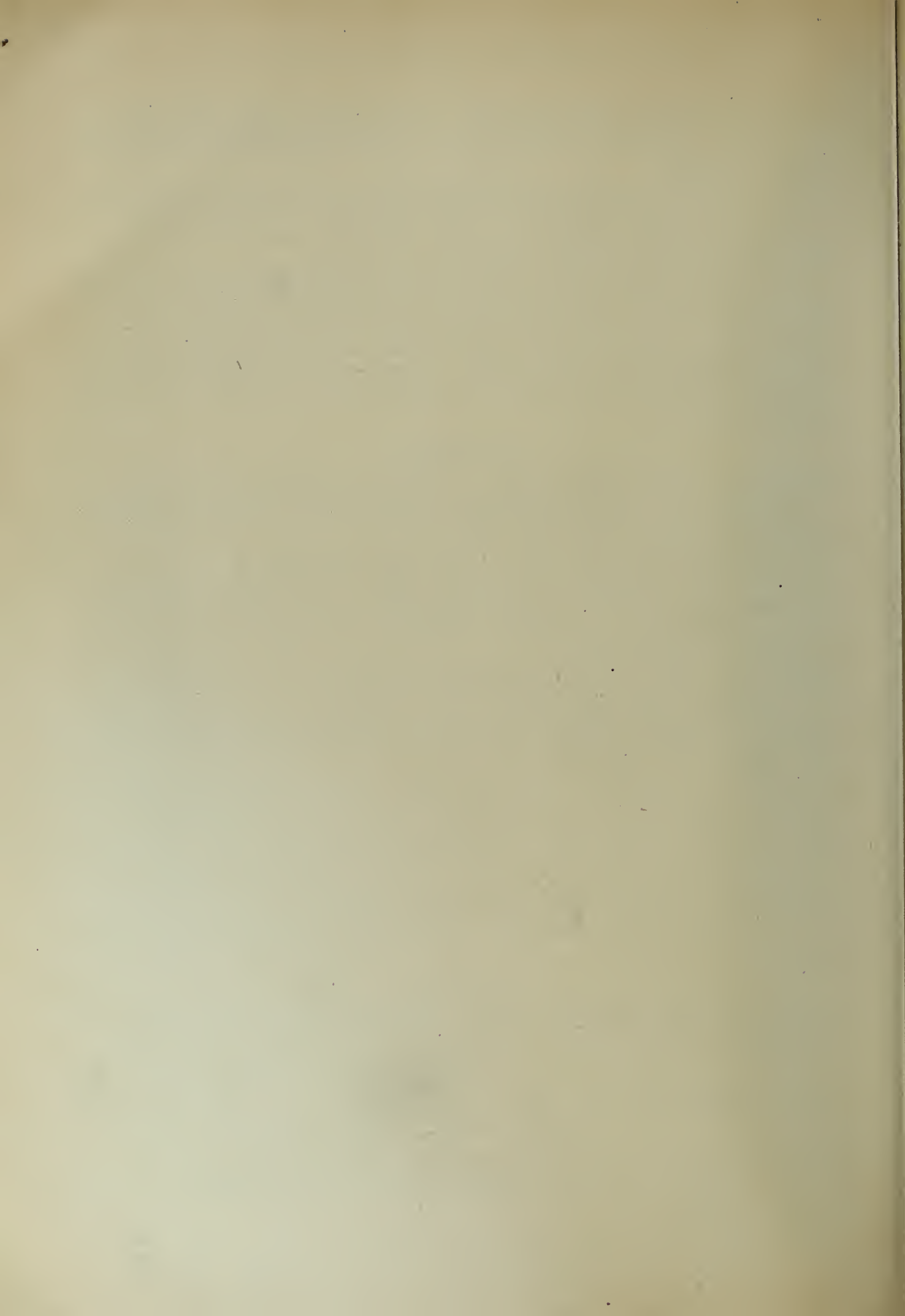
(5) The oral administration of the Shiga vaccine gives rise to less general reaction than does its inoculation subcutaneously.

(6) The antibody titre of the serum of rabbits immunised *per os* is appreciably raised by repeated ingestions.

(7) Attempts to secure, by subcutaneous and oral administration of killed vaccines, a solid active immunity against the potent soluble toxin of *B. dysenteriae* (Shiga) intravenously administered, have not been attended with the striking success realised in previous work when active immunity was sought to the live bacilli similarly administered. Consequently, comparative estimates of the value of oral and subcutaneous methods in this connection are not at present possible.

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A COMPARATIVE STUDY OF BOVINE ABORTION AND UNDULANT FEVER, FROM THE BACTERIO- LOGICAL POINT OF VIEW.

By Z. KHALED,

Bacteriologist, Hygienic Institute, Cairo.

(From the Bacteriological Department, Lister Institute, London.)

(With 2 charts.)

EARLY in 1914 Kennedy (1914) while testing some samples of goat's milk for agglutination of *B. melitensis*, found, to his surprise, that the control cow's milk gave a positive result. Following up this observation he noted that five out of the 13 specimens of cows' milk examined by him contained agglutinins for the organism of Malta fever.

Fabyan and Theobald Smith (1912) had shown that the tuberculous-like lesions produced by inoculating guinea-pigs with raw cow's milk were due to *B. abortus* Bang, the cause of bovine abortion. Since then other workers, notably Zwick and Krage (1913) confirmed the finding of *B. abortus* in the milk of infected cows, this excretion taking place irrespective of any lesion in the udder.

These facts remained uncorrelated until A. E. Evans (1918), in an illuminating piece of work, showed that *B. abortus* and *B. melitensis* were morphologically and serologically (agglutination) indistinguishable. Meyer, Shaw and Feusier (1920), later, corroborated Evans' views by a series of absorption tests.

It was for the purpose of further elucidating the relationship of these two organisms that the present investigation was undertaken.

The cultures used in this research were supplied by the National Collection of Type Cultures and are representative of strains isolated in America, on the Continent and in this country. In all, 13 strains of *B. melitensis*, 10 strains of *B. abortus* and 3 of *B. paramelitensis* were examined.

MORPHOLOGY AND NOMENCLATURE.

These three organisms are morphologically indistinguishable, occurring as small rods 3-5 μ in length with somewhat pointed ends. They are non-motile, stain uniformly with basic stains and are gram-negative. They are, however, somewhat pleomorphic and the same strain may show bacillary, cocco-bacillary or coccoid forms from time to time or the three forms may occur together in

the same culture. The age of the culture, the culture medium, and the method of staining have apparently nothing to do with this transformation.

The term "Micrococcus" is, therefore, inexact; and, though the term "Bacterium" would be correct, it would be better still if the generic name "Brucella," in honour of Sir David Bruce who discovered the first species (*melitensis*) in 1886, were adopted as suggested by Meyer and Feusier. Thus we would have *Brucella melitensis*, *Brucella abortus* and *Brucella paramelitensis*.

BIOCHEMICAL AND CULTURAL CHARACTERS.

The three organisms have the following common cultural and biochemical characters. They grow very slowly and scantily on ordinary agar, preferring glucose agar, on which medium they give a good growth after 36–72 hours' incubation at 37° C. The colonies are small, circular, with a smooth margin 2–3 mm. in diameter and whitish in colour. Later the growth tends to become confluent. In trypsin broth a uniform turbidity is produced after the third day without any surface pellicle. Litmus milk becomes alkaline and is not coagulated.

There is no change (acid or gas) in Hiss' serum with lactose, saccharose, dulcete, mannite or glucose, even after two weeks' incubation.

MODE OF INFECTION.

Infection with *B. abortus* may take place during copulation with males who have previously covered infected animals or who are themselves infected and excrete bacilli in their seminal fluid. The bedding may also carry infection, becoming contaminated with the vaginal discharges, amniotic fluid or foetal membranes from infected cows. Apparently the most usual mode of infection, however, is ingestion of food infected with amniotic fluid or afterbirth, as this ensures a considerably larger quantity of virus. After an incubation of 33–230 days a catarrhal condition of the genital passages with some discharge makes its appearance, and the secretion of milk is diminished. This is followed in 3–4 days by abortion accompanied by moderate pains and mild general manifestations. The animal recovers and then either remains sterile or aborts soon after service, generally after two months. The disease is highly infectious and rapidly spreads through whole herds affecting especially young animals. The males apparently act as carriers. The causal organism can be isolated from the spleen, liver, testes, seminal vesicles and uterine discharges as well as from the milk; the foetal membranes, stomach, amniotic fluid and cotyledons as a rule give pure cultures. Isolation and disinfection are very effective measures.

In undulant (Malta) fever, infection may occur through contamination of superficial scratches or pricks, though in the great majority of cases the disease is contracted by the ingestion of infected food—mainly goat's milk. It is well to mention, however, that cases have been reported in which infection was

In goats the disease may be present without producing any obvious clinical manifestations.

Thus the two diseases present the picture of a bacteraemia, with a close similarity in the modes of infection, and of excretion of the causal organism.

Anti-sera were prepared against the three organisms, *B. abortus*, *B. melitensis* and *B. paramelitensis* and the 30 selected strains tested against these sera by means of the agglutination and absorption reactions.

The various cultures are referred to by their number only in the tables which follow, and for sake of brevity the findings obtained with nine only of the 30 strains are recorded. The results obtained with the other 21 strains differed in no respect from those given below.

Cultures of *B. paramelitensis*. Nos. 82 and 84.

Cultures of *B. abortus*. Nos. 624, 830, 895 and 900.

Anti-melitensis serum (titre 1 : 6400) tested against the various strains.

Strain	Dilution of serum								Control
	1 : 100	1 : 200	1 : 400	1 : 800	1 : 1600	1 : 3200	1 : 6400	1 : 12800	
78	+++	+++	+++	+++	+++	+++	+	-	-
80	+++	+++	+++	+++	+++	+++	+++	-	-
893	+++	+++	+++	+++	+++	+++	+++	-	-
82	+	-	-	-	-	-	-	-	-
84	+	-	-	-	-	-	-	-	-
624	+++	+++	+++	+++	+++	+++	++	-	-
830	+++	+++	+++	+++	+++	+++	++	-	-
895	+++	+++	+++	+++	+++	+++	++	-	-
900	+++	+++	+++	+++	+++	+++	+	-	-

Table II.

Anti-abortus serum (900) tested against the various strains.

Strain	Dilution of serum								Control
	1 : 100	1 : 200	1 : 400	1 : 800	1 : 1600	1 : 3200	1 : 6400	1 : 12800	
624	+++	+++	+++	+++	+++	+++	++	-	-
830	+++	+++	+++	+++	+++	+++	++	-	-
895	+++	+++	+++	+++	+++	+++	+++	-	-
900	+++	+++	+++	+++	+++	+++	+++	-	-
78	+++	+++	+++	+++	+++	++	-	-	-
80	+++	+++	+++	+++	+++	+++	-	-	-
893	+++	+++	+++	+++	+++	++	++	-	-
82	+++	+++	+++	+++	+	-	-	-	-
84	+++	+++	+++	+++	+++	+	-	-	-

Table III.

Anti-paramelitensis serum (84) tested against the various strains.

Strain	Dilution of strain								Control	
	1 : 100	1 : 200	1 : 400	1 : 800	1 : 1600	1 : 3200	1 : 6400	1 : 12800		
82	+	+	+	+	+	+	+	+	-	-
84	+	+	+	+	+	+	+	+	-	-
78	+	+	+	-	-	-	-	-	-	-
80	+	+	+	-	-	-	-	-	-	-
893	+	-	-	-	-	-	-	-	-	-
624	+	+	+	-	-	-	-	-	-	-
830	-	-	-	-	-	-	-	-	-	-
895	+	-	-	-	-	-	-	-	-	-
900	+	-	-	-	-	-	-	-	-	-

It will thus be seen that agglutination alone helps us little in arriving at any valid conclusions as to the serological relationship of *B. melitensis* and *B. abortus*, and a series of absorption tests was made, therefore, to determine this point.

Meyer, Shaw and Feusier (1920), in their paper quoted above, divided the organisms of undulant fever and cattle abortion into four groups serologically, their Group IV containing *B. paramelitensis* only. The results of the following absorption tests, however, do not lend support to this method of classification and justification is therefore felt in mentioning them in some detail.

TECHNIQUE OF ABSORPTION USED.

Antigens. The 48-hours' growth on 1 per cent. glucose agar slopes was washed off with sterile distilled water, using 0.5 c.c. for each culture.

The antisera, prepared as above were diluted 1 : 25 with salt solution (0.75 per cent.).

To the emulsion obtained from several slopes was added an equal volume of the diluted serum, giving a final dilution of 1 : 50 of the serum. The mixture was then incubated for two hours, centrifuged and the clear serum pipetted off.

The absorption was considered as satisfactory when all agglutinins for the absorbing strain had been removed.

It may be mentioned that broadly speaking, a full agar slope is practically always found sufficient to absorb all the specific agglutinins from 1 c.c. of 1 : 50 serum whose titre is 1 : 6400 (or less) in 2 hours. This obviates the subsequent absorption which is liable to become necessary if a smaller amount of organisms is used.

The technique of the agglutination experiments performed with the absorbed sera was the same as that already described earlier in this communication.

Only the more important results are recorded.

Table IV.

Anti-melitensis serum absorbed with *B. melitensis* (80) and tested against strains 80, 893, 830, 895 and 900.

Strain	Dilution of serum				Control
	1 : 100	1 : 200	1 : 400	1 : 800	
80	—	—	—	—	—
893	—	—	—	—	—
830	—	—	—	—	—
895	—	—	—	—	—
900	—	—	—	—	—

The same result was obtained after absorption with any other strain of *B. melitensis*.

Table V.

Anti-melitensis serum (80) absorbed with *B. abortus* (900) and tested against strains 80, 893, 900, 895 and 830.

Strain	Dilution of serum				Control
	1 : 100	1 : 200	1 : 400	1 : 800	
80	—	—	—	—	—
893	—	—	—	—	—
900	—	—	—	—	—
895	—	—	—	—	—
830	—	—	—	—	—

The same result was obtained after absorption with any other strain of *B. abortus*.

Table VI.

Anti-abortus serum (900) absorbed with strain 900 and tested against strains 78, 80, 893, 830, 895 and 900.

Strain	Dilution of serum				Control
	1 : 100	1 : 200	1 : 400	1 : 800	
78	—	—	—	—	—
80	—	—	—	—	—
893	—	—	—	—	—
830	—	—	—	—	—
895	—	—	—	—	—
900	—	—	—	—	—

The same results were obtained if *abortus* strains other than the homologous were employed or other anti-abortus sera used.

Table VII.

Anti-abortion serum (900) absorbed with *B. melitensis* (80) and tested against strains 78, 80, 893, 830, 895 and 900.

Strain	Dilution of serum				Control
	1 : 100	1 : 200	1 : 400	1 : 800	
78	—	—	—	—	—
80	—	—	—	—	—
893	++	+	—	—	—
830	+++	+++	+++	+++	—
895	+++	+++	+++	+++	—
900	+++	+++	+++	+++	—

Other anti-abortion sera absorbed with this as well as other strains of *B. melitensis* gave similar results.

The salient features of these absorption tests can be summarised as follows:

1. When an anti-melitensis serum is absorbed with *B. melitensis* all agglutinins for *B. melitensis* and *B. abortus* are removed.
2. The same result is obtained if *B. abortus* is used to absorb an anti-melitensis serum.
3. Anti-abortion serum absorbed with any *abortus* strain loses all agglutinins for both *B. melitensis* and *B. abortus*.

4. Anti-abortion serum absorbed with *B. melitensis* has lost its power to agglutinate *B. melitensis* strains but still agglutinates *B. abortus* to full titre.

From these results it would appear that *B. melitensis* is a sub-strain of *B. abortus*, in the sense employed by Schütze (1922).

Absorption experiments on the same lines with *B. paramelitensis* have not so far been carried out.

PATHOGENICITY.

The close morphological, bio-chemical and serological relationship between *B. melitensis* and *B. abortus* at once raises the question of their relative pathogenicity. This becomes much the more important if we consider the fact that 25 per cent. of the milch cows in this country are infected with *B. abortus*, and this percentage is even higher on the Continent and in America. These bacilli are found even in the "certified milk." A series of animal experiments on guinea-pigs, goats and monkeys was carried out, a few of which will be mentioned.

Guinea-pig 4. Intraperitoneal inoculation of $\frac{1}{4}$ -slope culture (48 hrs.) of *B. abortus* (900), 17. ii. 21. In 24 hours the temperature began to rise and reached 104° in 36 hours. The fever continued for three weeks, reaching its highest point (105° F.) at the end of the second week. The thermometer gave a higher reading in the evening (5 p.m.) than in the morning (10 a.m.). The animal looked sluggish and was disinclined to feed during the first week after injection. It was killed on March 16th.

Post-mortem. No marked congestion or exudate in peritoneum. Spleen enlarged and adherent to diaphragm with a small abscess between superior border and the diaphragm. Liver slightly congested. Kidney normal; testes and epididymis normal. Lumbar and mesenteric glands enlarged. No change was noted in heart or lungs. The chief histological finding was an increase of lymphoid tissue in the spleen.

The organism was recovered in pure culture from the spleen, liver, splenic abscess and mesenteric glands.

Guinea-pig 5. Received $\frac{1}{4}$ -agar slope of *B. abortus* (strain 830).

This animal gave findings similar to those recorded in the case of Guinea-pig 4, with the exception that the temperature reached 105° F. on the third day after inoculation and that it began to drop to normal about the end of the second week.

Guinea-pig 7. Inoculated with *B. abortus* (strain 895). Similar rise in temperature, reaching 105° F. on the third day and returning to normal on the nineteenth day of the disease.

In both cases (Guinea-pigs 5 and 7) the same post mortem and histological findings were obtained. *B. abortus* was recovered from the kidney in No. 5 and from the bone marrow of No. 7. Cultural examination of the heart blood gave negative results in both cases.

Guinea-pig 8. Inoculated with *B. melitensis* (strain 893) ($\frac{1}{4}$ -slope, 48 hours' culture).

The temperature in this case remained high (105–106° F.) for the first ten days, the pyrexia continuing for three and a half weeks.

Post-mortem findings similar to the above three except that splenic and hepatic congestion was more marked.

In order to measure the relative pathogenicity of the two organisms to guinea-pigs, animals of about equal weight were inoculated intraperitoneally with $\frac{1}{4}$, $\frac{1}{2}$ and 1 slope of *B. melitensis* and with 1, 2, $2\frac{1}{2}$ and 3, etc. slopes of *B. abortus*. It was found that $\frac{3}{4}$ of a slope of *B. melitensis* killed a guinea-pig of 240 grms. in 18 hours. To kill a guinea-pig of the same weight in approximately the same time $4\frac{1}{2}$ slopes of *B. abortus* were required. The amount of growth per slope in both cases was practically equal, thus showing that *B. melitensis* is about six times more virulent than *B. abortus* for the guinea-pig. The method is admittedly somewhat crude, since the number of organisms in the M.L.D. has not been determined, but it gives some idea of their comparative pathogenicity.

The following experiment on goats was then carried out:

Two goats *A* and *B* were examined for previous *melitensis* infection. The blood and urine were examined culturally and the serum tested for the presence of agglutinins for *B. melitensis* and *B. abortus*. All tests proved negative.

Goat A then received one 48 hours agar slope culture of living *B. abortus* (900) intravenously (jugular) on 25. iv. 21.

Goat B received a similar dose of *B. abortus* (895) on the same day.

The blood was examined culturally from time to time, a positive result being obtained 48 hours after the injection (only few bacilli). Later, repeated cultural examination on different occasions gave uniformly negative results. The goats showed very little general reaction. They stood the inoculation well, partook of their food as usual and in fact their general health did not apparently suffer. Urine examinations proved negative throughout.

The antibody response to this inoculation was most marked. Thus the agglutinins in the blood of Goat A for *B. abortus* (900) were:

Date	Titre
3. v. 21	1 : 400
10. v. 21	1 : 1600
17. v. 21	1 : 3200
24. v. 21	1 : 6400
4. vi. 21	1 : 6400
11. vi. 21	1 : 12800

Date	Titre
22. vi. 21	1 : 25600
29. vi. 21	1 : 25600
5. vii. 21	1 : 12800
12. vii. 21	1 : 12800
19. vii. 21	1 : 6400

The agglutination titre for *B. melitensis* was somewhat less (1 : 6400–1 : 12800) and least of all for paramelitensis strains (1 : 6400 at most).

Absorption tests carried out with this serum were rather troublesome, as in most cases at least 3 or 4 slopes were required to completely absorb $\frac{1}{2}$ c.c. of 1 : 25 dilution of the serum. However, the same results were obtained with this goat's serum as with rabbits' sera mentioned above (see Tables VI and VII).

Unfortunately the goats available on this occasion were not pregnant and, therefore, the phenomena of abortion, the excretion of bacilli in the milk, and the presence of agglutinins in this latter secretion could not be demonstrated.

CROSS IMMUNISATION.

An attempt was made to find out whether previous immunisation of monkeys with *B. abortus* could ward off a subsequent melitensis infection. The result is of interest and deserves detailed mention:

1. *Macacus rhesus*. Received three doses ($\frac{1}{4}$, $\frac{1}{2}$ and $\frac{1}{2}$ slope of killed *B. abortus*) intravenously at intervals of ten days. The serum finally agglutinated *B. abortus* 1 : 6400 and *B. melitensis* 1 : 3200.

2. *Macacus sinicus*. Not previously immunised, to act as control.

Both monkeys were inoculated on July 1st with $\frac{1}{2}$ -slope of *B. melitensis* intravenously (living culture).

Both stood the infection fairly well for the first 48 hours, and then changes began to appear. The Rhesus (No. 1) continued to take his food and to play as usual, whereas the Sinicus (No. 2 control) became dull, weak, lazy and was inclined to scratch his forehead and to pull some of his crown hair, as if suffering from headache.

At the end of the first week the serum of No. 1 showed a higher titre for *melitensis* (1 : 6400) and *abortus* (1 : 10000) and the blood culture was negative. The second monkey gave agglutination in 1 : 800 and the blood culture was positive (scanty growth).

The accompanying temperature charts show the febrile reaction of *M. sinicus* (No. 2 control) reaching as high as 105.5° F., whereas *M. rhesus* (No. 1) did not show any rise beyond 103.5° F., and this on one occasion only (sixth day).

As regards weight the following table shows that, whereas both monkeys lost weight immediately after the inoculation, the Rhesus monkey soon returned to normal, whereas the control monkey continued to lose weight:

	<i>M. rhesus</i> (No. 1)	<i>M. sinicus</i> (No. 2 control)
Before inoculation	2500 gms.	1950 gms.
3. vii. 21	2480	1910
6. vii. 21	2450	1900
9. vii. 21	2330	1800
20. vii. 21	2500	1700
1. viii. 21	2530	1560

The experiment shows quite clearly that the immunisation of Monkey No. 1 (Rhesus) with *B. abortus*, had been able to protect that monkey against an infecting dose of *B. melitensis*, which in the control non-immunised Monkey No. 2 (Sinicus) produced a quite definite febrile illness.

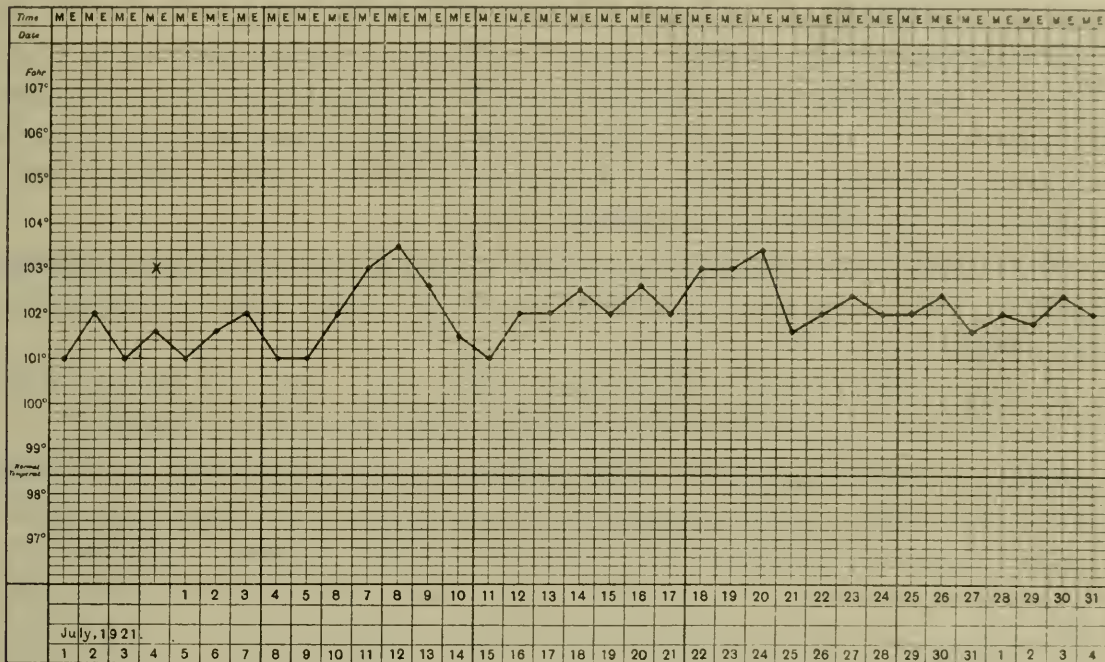


Chart 1. *M. rhesus* ♀ immunised with *B. abortus* and afterwards infected with *B. melitensis*.

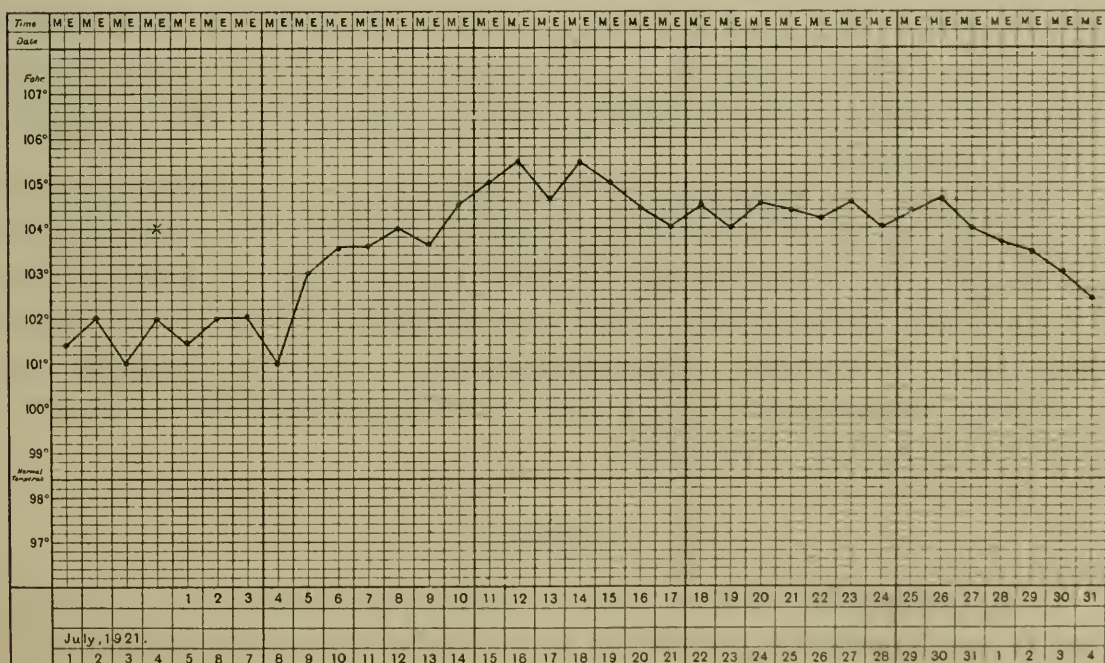


Chart 2. *M. sinicus* ♂ control (non-immunised) infected with *B. melitensis*.

PATHOGENICITY TO MAN.

Naturally the question crops up whether or not *B. abortus* being so closely related to *B. melitensis*, is capable of producing an undulant or other form of fever in man. In this connexion I repeat the words of Kennedy (1914): "I think the possibility of melitensis infection of cows in this country should not be lightly thrust aside. I have heard of two cases of undulant fever in people who have never been out of England and it is possible that there are others undiagnosed." I have myself seen cases in Egypt which have never had a chance of ingesting goat's milk and yet suffered from typical melitensis fever as confirmed by laboratory diagnosis.

Meyer and Fleischner (1920) were able to produce in monkeys an undulant-like fever with, in some cases, a fatal result, by means of *B. abortus* (feeding and inoculation).

Cooledge (1916) found anti-abortus bodies in the serum of human beings fed on milk from cows which were suffering from contagious abortion.

There is, however, the fact that undulant fever is unknown in countries where goat's milk is not an important article of food, even though contagious abortion may be widespread. This seems to me more apparent than real. The geographical distribution of undulant fever has been steadily widening since 1886 when it was first known to be a definite clinical entity with a specific organism. Before that time it was often mistaken for a transient fever, for typhoid or for early phthisis, having a very variable symptomatology. The low virulence of *B. abortus* as compared with *B. melitensis* brought out in the experiments on guinea-pigs, would indicate that a larger dose would be required to infect; but, apart from this, there is no reason why *B. abortus* should not produce a febrile condition. In this respect the possibility of the ingestion of large quantities of milk producing a passive immunity to *B. abortus* might require consideration.

Whether the two organisms are one and the same or not and whether the lowered virulence and the different behaviour of *B. abortus* in the absorption tests are produced by passage through cows, I am not ready, at present, to say. It may be that *B. abortus* bears the same relation to *B. melitensis* as cow-pox to small-pox. The fact that cross immunisation of monkeys is successful seems to enhance this supposition, but it is, of course, inadvisable to draw conclusions from a single experiment.

SUMMARY.

1. Morphologically *B. abortus* and *B. melitensis* are identical. The "coccoid" form is not a constant feature and a more satisfactory generic name would be "Brucella."

2. The organisms cannot be differentiated by cultural, bio-chemical, or staining methods, or by the agglutination reaction.

3. From absorption experiments, it would appear that *B. melitensis* is a sub-strain of *B. abortus*.

4. Dose for dose *B. abortus* is much less virulent for the guinea-pig than *B. melitensis*, approximately about 1 : 6.

5. Immunisation of monkeys (one experiment only) with killed suspensions of *B. abortus* protected against subsequent infection with *B. melitensis*.

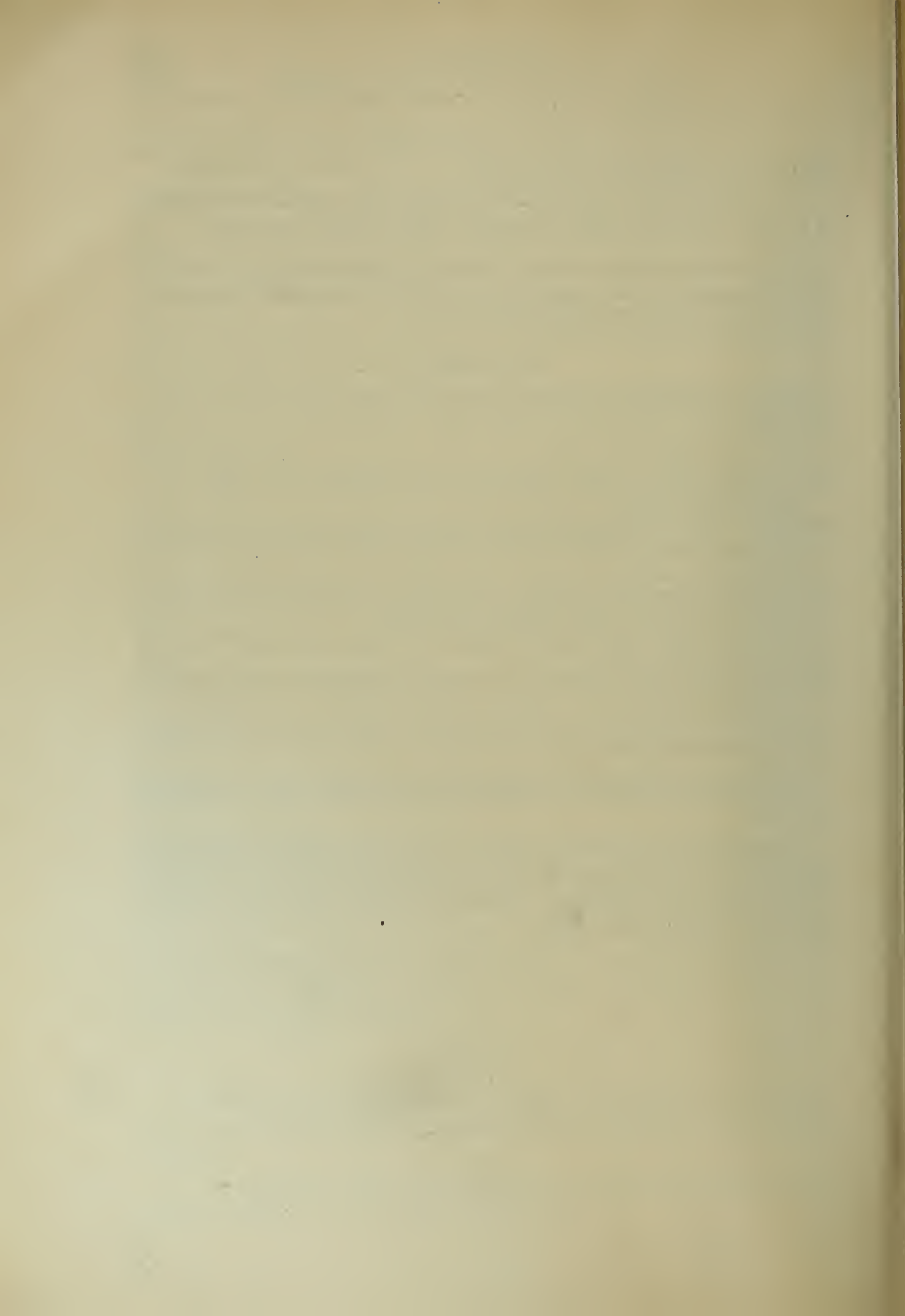
In conclusion I wish to thank Professor Ledingham for much valuable advice throughout the investigation, and Dr R. St John Brooks for supplying me with cultures from the National Collection.

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Only works to which I have referred in the text of the paper are included in this list.

The Reports of the Royal Commission for the study of Mediterranean Fever (1905–1907) as well as the reports of the Departmental Committee of the Board of Agriculture and Fisheries on Epizootic abortion (1909–1910) contain a great deal of useful information.



THE PRODUCTION OF PURPURA IN BIRDS AND AMPHIBIA BY INOCULATION WITH THROMBOCYTE ANTI-SERA.

By J. C. G. LEDINGHAM and H. M. WOODCOCK.

From the Lister Institute.

Certain peculiarities of bird blood such as the presence of nucleated elements known as thrombocytes, the absence of elements morphologically similar to the mammalian platelets, and retarded power of clotting, have long been recognised. The behaviour of these thrombocytes in shed blood, particularly their rapid clumping and tendency to disruptive changes, has suggested to many workers in the past the view that their function in the mechanism of coagulation is analogous to that of the mammalian platelet in spite of the morphological dissimilarity. In the light of previous experience with experimental purpura in mammals it was resolved to test whether support for this hypothesis, hitherto based on morphological considerations only, could be obtained experimentally.

From citrated or oxalated pigeon blood thrombocyte emulsions were prepared by a process of fractional centrifugalisation.

Owing to the small number of thrombocytes in pigeon blood relatively to that of platelets in mammalian blood (the normal pigeon was found to possess about 40,000 thrombocytes per cub. mm., whereas the guinea-pig, *e.g.*, possesses about 600,000), the difficulty of securing a sufficiency of thrombocytes for immunisation purposes was very great and was intensified by the fact that gravity helped so much less than in the case of the platelet. In spite of these difficulties, however, rabbits were immunised with thrombocyte emulsions freed as far as possible from other elements. The sera from these animals were tested *in vitro* for agglutinating action on bird blood elements, and also *in vivo* to ascertain whether evidence of selective action on the thrombocytes with consequent purpuric manifestations would be forthcoming. The results supplied such evidence. Subcutaneous injection of the antithrombocyte serum was followed in twenty-four hours by a local purpuric eruption which cleared up in five or six days.

Intraperitoneal inoculation of the serum caused a more rapid production of purpura confined, so far as naked-eye examination went, to the vascular mesenteric tissue and to the fatty tissue surrounding the gizzard.

Skin purpura was present only in the neighbourhood of the initial puncture. Blood examinations carried out during the period following inoculation yielded interesting and confirmatory data pointing to selective action on the thrombocytes. The red cells were estimated in the usual way. On stained films the thrombocytes were counted relatively to the reds. In the red-cell chamber (without lysis) leucocytes and thrombocytes were counted together, and the difference between the figure so obtained and that found for the thrombocytes yielded a figure for the leucocytes. The hæmatoblasts or large primitive cells—the probable precursors of the thrombocytes and also perhaps of the erythroblasts—were counted on stained films relatively to the red cells.

Thrombocytes and hæmatoblasts fell in twenty-four to forty-eight hours to minimal numbers, and the fall in each case was followed by gradual rise to

figures exceeding the normal. Within ten days the normal formula was usually established. One illustrative example may be cited.

(The normal pigeon possesses a little over 4,000,000 reds per cub. mm., about 30,000 leucocytes, and about 40,000 thrombocytes. The hæmatoblasts were usually under 1000 per cub. mm.)

Pigeon received 3 c.c. Antithrombocyte Serum subcutaneously on 2nd February.

	Thrombocytes.	Hæmatoblasts.	R. B. C.
Before injection . . .	46,000	1,000	4,340,000
1st day . . .	8,072	0	4,440,000
2nd „ . . .	0	3,000	4,470,000
3rd „ . . .	3,828	9,000	5,360,000
5th „ . . .	35,076	5,000	4,560,000
6th „ . . .	52,600	2,000	5,260,000

In frogs encouraging results have also been obtained, but in these animals the difficulty of securing strong emulsions of thrombocytes (or “spindle-cells”) has not yet been completely overcome.

N^o 14

THE RECORD OF A BRIEF EXPERIENCE WITH THE SACHS-GEORGI TEST.

BY

P. PARTHASARATHY, L.R.C.P., L.R.C.S. EDIN.,
D.P.H. CAMB., D.T.M. LOND.;

AND

MARY M. BARRATT, M.B., CH.B. GLASG.

*With an Historical Account of the Development of Flocculation
Tests for Syphilis,*

BY

J. C. G. LEDINGHAM, C.M.G., M.B., CH.B., F.R.S.

(From the Lister Institute, London.)

OUR only excuse for placing the following notes on record is the fact that hitherto comparatively few collated results of parallel Wassermann and Sachs-Georgi tests have appeared from laboratories in this country. It will doubtless be the policy of the near future to institute carefully planned investigations on the great scale dealing with parallel complement-fixation and flocculation tests with a view to the selection of the best working methods and the correlation with clinical experience of what may prove to be the future routine serological test for syphilis. Pending the appearance of such massed statistics it may be of interest to record the capabilities of a flocculation test performed with material sent from outside sources to the diagnosis department of this institute accompanied by clinical data of varying completeness. Our series comprises only 265 serums forwarded to the institute during the period July to October, 1921. One of us (B.) carried out the routine Wassermann tests, while the other (P.) undertook, solely as a matter of research, the parallel flocculation tests.

METHODS EMPLOYED.

Flocculation Tests.

Two antigens were employed throughout ("D" and "L"), and the results with both are summarized. Antigen "D" was that of Bordet and Ruelens (1919), with the addition of cholesterol as recommended by Dreyer and Ward (1921), and the latter's technique was strictly adhered to so far as qualitative diagnosis was concerned, no attempt being made in this short series to probe the possibilities of quantitative evaluation. A dropping apparatus was used, and for the first 122 serums five tubes containing dilutions of serum varying from 1 in 1.25 to 1 in 26.4 in a total

volume of 25 drops were put up. Later, as the results from the first tube were indefinite, and as a large quantity of serum was required, the first tube was dispensed with. The mixture of antigen, saline, and serum was incubated at 37° C. for seven hours in a water-bath, and the results then read. One batch of antigen was employed throughout, and was preserved in a cool incubator at a temperature of about 22° C. Antigen "L" was simply an alcoholic extract of guinea-pig's heart muscle, to which was added a 1 per cent. solution of cholesterin in the proportion of 9-parts of heart extract to 1 of the cholesterin solution.

For the first 132 cases four tubes were put up, containing in a total volume of 26 drops a constant quantity of pure serum (6 drops) and 20 drops of a dilution of antigen varying from 1 in 20 to 1 in 80. For the remaining half of the series the antigen was kept constant in the four tubes (20 drops of a 1 in 40 suspension) and the serum was varied from 1 drop to 6, the total volume being made up to 26 drops.

The dilutions of serum in the tubes were therefore 1 in 3, 1 in 6.5, 1 in 13, and 1 in 26. The serums had been previously heated at 54° C. for 90 minutes. The tubes were incubated for five hours in a water-bath at 37° C. and the results were read 20 minutes after removal from the bath. They were reread after standing at room temperature for twelve to fourteen hours.

One batch of this antigen "L" was used throughout and experience showed that preservation in the cold (about 4° C.) was most satisfactory. Results with both antigens were recorded as "standard" (easily visible to the naked eye), "trace" (visible with lens), and "negative." At each test controls were used—namely, one Wassermann-positive serum, one Wassermann-negative serum, also previously tested positive and negative Sachs-Georgi samples. The antigen was also put up alone to exclude any non-specific precipitation.

Wassermann Tests.

These were performed with the antigen employed here—namely, an alcoholic extract of guinea-pig's heart muscle, to which is added before the test a 1 per cent. cholesterin solution in the proportion of 5 parts of extract to 4 parts of the cholesterin solution. Of this antigen a 1 in 90 dilution was used.

RESULTS.

Total number of serums tested	265
Of these, Wassermann positive	91
Positive flocculation ("L")	86
Positive flocculation ("D")	74

The degree of agreement may be otherwise expressed for the two antigens used, thus:

W+ L+	83	W+ D+	71
W- L-	171	W- D-	171
W+ L-	8	W+ D-	20
W- L+	3	W- D+	3
Total	265	Total	265

Thus, comparing the Wassermann and the flocculation tests by "L" antigen the total number of discrepancies in the series was 11, or 4.1 per cent., and in the case of "D" 23, or 8.6 per cent. The 91 positive Wassermann tests are classified as 70 "full positive," 10 "partials," and 11 "slight partials." Corresponding to this grouping the results with "L" and "D" are as follows:

	Total Positive.	Full Positive.	Partial Positive.	Slight Partial.
W	91	70	10	11
"L"	83	67	9	7
"D"	71	61	5	5

Thus 5 of the 8 missed positives with "L" antigen correspond to partial and slight positive Wassermann reactions, and half of those missed by "D" antigen are similarly accounted for. Only three serums yielded positive results with both "L" and "D" antigens,

while the corresponding Wassermann tests were negative. In all three the flocculation tests were recorded as "trace" reactions. One was from a latent case under treatment and in which the Wassermann test had been positive seven months previously; another was untreated and was stated to have a rash on trunk and perforation of palate (certainly a suggestive history); while the third came with no particulars.

Treated and Untreated Cases.

One hundred and fifteen of the series were stated to be treated cases, while 150 were entered as untreated or "no particulars." They are thus grouped:

			Total.		W+.	"L"+.	"D"+.
Treated	115	31	28
Untreated	150	60	55
							48
Total	265	91	83
							71

Primary Cases.—There were 20 cases of primary sore, in 9 of which spirochaetes were found. They yielded 9 positive results by all three methods.

Only 5 cases of nerve syphilis appear in the list, with a yield of one positive result with the Wassermann test, the corresponding flocculation tests proving negative.

Discussion.

It has been customary to take the discrepancy percentage as a measure of the efficiency of a flocculation test, and on this basis the results from our small series compare favourably with those recorded by other workers in this country who have published results within the last eighteen months. The discrepancy percentage, however, must in the meantime be regarded solely as a provisional measure of efficiency. The ultimate interpretation of discrepancies between complement-fixation and flocculation tests must, as Lesser (1919) has wisely pointed out in a paper dealing with results by three different methods (Wassermann, Meinecke, and Sachs-Georgi), rest with the clinician, whose record of the past, present, and future clinical course of the case and response to treatment will materially assist the serologist to appraise with some confidence the results from different methods and to eliminate those which prove unsatisfactory. The flocculation test may indeed prove, as Dreyer and Ward suggest, to fit the clinical facts even better than the Wassermann. Even now, with some sixteen years' experience of the Wassermann test, doubtful reactions are constantly occurring (especially in treated cases), and the correlation of these with parallel flocculation tests may serve to place the latter on a proper footing with respect to the clinical condition in the diagnosis of which it is designed to participate. So far, the agreement between Wassermann and flocculation tests carried out under the best auspices is remarkably good, and there is every reason to expect that further extensive experience of parallel tests in relation to syphilitic disease will lay a sure foundation for the flocculation test as the future test of election for syphilis.

THE DEVELOPMENT OF FLOCCULATION METHODS.

It is, we think, of prime importance that the profession should be familiar with the historical development of a test

which, now that the time seems favourable, may have a chance of establishing itself as the method of choice.

The Wassermann reaction dates from 1906, when the paper by Wassermann, Neisser, and Bruck appeared. This test, which at first was regarded as a true antigen-antibody reaction, was made possible by the demonstration in 1901 by Bordet and Gengou that, in the reaction between antigen and antibody, complement, if present, was fixed and consequently rendered unavailable for completion of haemolysis in a haemolytic system. In the following year (1907) it was shown by Landsteiner, Müller and Pötzl, Levaditi and Yamanouchi, and Porges that the effective substance which reacted with syphilitic serum was not present solely in organ extracts of luetic cases, but also in extracts of normal organs. Further, this effective substance was shown by them to be contained almost solely in the alcohol-soluble fraction, its association therefore with a lipoidal substrate being thus demonstrated. In this same year Michaelis noted in one single instance the formation of a visible precipitate when the extract of a luetic liver was brought in contact with a luetic serum. He concluded wrongly that this observation supported Wassermann's view of the specific character of the Wassermann test as a true antigen-antibody reaction. However, chance observation as it was, it gave the impetus to those attempts which have followed each other down to the present time to substitute for the complicated system of the Wassermann reaction a straightforward flocculation test involving two ingredients only. Following this isolated observation of Michaelis, Porges and Meier (1907, 1908) were the first to probe the possibilities of practical application, and, curiously enough, they chose to work, not with alcoholic organ extracts, but with commercial lecithin preparations (from egg yolk), the capacity of which to act as antigen in the Wassermann reaction had already been demonstrated. For many reasons, however, and more especially the instability of the lecithin suspensions and the occurrence of flocculation with normal serums, the use of lecithin was discontinued, and in later work by Porges, in collaboration with Elias, Neubauer, and Salomon (1908), resort was had to the use of sodium glycocholate, a salt possessing colloidal properties in solution, and which Levaditi and Yamanouchi had previously found to function as an antigen in the Wassermann test. Porges states that with this salt as antigen results were achieved which approximated in accuracy to those yielded by the Wassermann test; but it would appear that no thorough testing of flocculation methods was possible or practicable at this period, the Wassermann test in all its many modifications and improvements claiming the undivided attention of serologists and clinicians alike.

In the meantime the properties and mechanism of action of lipoids in serological reactions were being minutely investigated by numerous workers. Among them Sachs and Rondoni (1909), in the course of a study of the antigenic powers of lecithin in the Wassermann reaction, demonstrated an increased action of lecithin in the presence of sodium oleate.

and in the following year (1910) Browning, Cruickshank, and McKenzie demonstrated the remarkable action of cholesterin in increasing the amount of complement absorbed in the presence of a mixture of lecithin and luetic serum. Taking advantage of this discovery, Herman and Perutz (1911) mixed cholesterin with sodium glycocholate with the object of obtaining a more visible flocculum in the presence of syphilitic serum. This modification proved in Herman and Perutz's hands more sensitive than the original technique of Elias, Neubauer, Porges, and Salomon with the glycocholate alone, and yielded positive results in certain luetic cases when the original method gave no visible flocculation whatever. Meanwhile the flocculation of these lecithin suspensions again drew attention to the study of the inner mechanism of the Wassermann reaction. Was the complement really fixed by virtue of a precipitin reaction? No visible precipitation, apart from chance observation, was noted, which might account for the fixation of complement in the presence of alcoholic organ extract and luetic serum. Jacobsthal (1910), however, succeeded in demonstrating by the ultra-microscope the occurrence of floccular masses, the development and growth of which could be followed. He even attempted to employ the method practically, and stated that absolutely negative results occurred with non-luetic serums, but the percentage of positive results was not quite so high as in the Wassermann-positive series.

This work was followed up by Bruck and Hidaka (1910), who found that, by the aid of the centrifuge, precipitates, small indeed in bulk, could be obtained after some prolonged contact of alcoholic liver extract from foetal lues with luetic serum at a low temperature. With the object of making this precipitate more bulky, and consequently more visible, they added mastix to the system, and with this technique it was possible to obtain results approximating to the Wassermann, but a fair percentage of discrepancies occurred. The outcome of this form of study was to support strongly the view that the Wassermann reaction depends primarily on fixation of complement by an invisible precipitate (nascent precipitate) taking place in the earliest stage of the interaction between the alcoholic organ extract and the luetic serum. Apparently the only difficulty now was to secure an extract sufficiently sensitive to show visible precipitation in the presence of luetic serum. Hecht (1916) had definitely successful results, but the most recent and definitive stage in the development of flocculation methods as practical diagnostic aids was reached towards the end of the war, when Meinecke (1917) and Sachs and Georgi (1918) came forward with tested procedures, the latter of which (by Sachs and Georgi) is likely, either in its original or more possibly in some modified form, to become the method of election.

Meinecke's method is known as the two-stage method, and in its ordinarily practised form (the so-called salt method) is carried out on the following lines, purely technical details being omitted :*

* In flocculation procedures technical details are all-important, but those interested must refer to the original papers cited below.

An ordinary Wassermann extract in distilled water dilution, and containing no cholesterin, acts as antigen. Mixtures of inactivated serum and antigen in this salt-free medium are kept at incubator temperature for twenty-four hours. At the close of this period all tubes should show flocculation, whether they contain normal or luetic serum. The second stage now commences. A definite amount of a sodium chloride solution of previously determined strength is run into each tube. After an hour's further sojourn in the incubator the tubes are read, when it is found that in the Wassermann-negative series the flocculi are dissolved up, while the Wassermann-positive series remain permanently flocculated.

The salt concentration determined from previous experiment is that which will in one hour just completely dissolve the flocculi in the normal controls, so that differences can be elicited as between strongly positive, weakly positive, doubtful, and negative series.

The Meinecke reaction appears to be a perfectly straightforward and feasible test. It has been tried by many workers in Germany, generally in conjunction with the Wassermann and with the Sachs-Georgi reaction presently to be described. On the whole, however, it has found much less favour than the latter.

With their experience of complement-fixation technique, and their intimate knowledge of the comparative values of simple alcoholic organ extracts and purified lipoids as antigens, Sachs and Georgi (1918) chose as antigen an alcoholic extract of bullock's heart (hearts of other animals are, however, admittedly suitable), to which cholesterin as an absolutely indispensable adjunct is added. For the Wassermann test it is of course not indispensable, though sufficiently advantageous to make its use almost universal, but for the flocculation test its presence is essential for visible precipitation. The cholesterinized alcoholic extract is diluted with five parts of physiological saline. One c.cm. of a 1 in 10 dilution of the inactivated patient's serum is mixed with 0.5 c.cm. of the diluted extract along with the necessary controls. The tubes are left either for two hours at 37° C., and then overnight at room temperature, or they are kept continuously at 37° C. for eighteen to twenty hours. Positive reactions are read as + + +, + +, and +, while the control serums remain clear or slightly opalescent. Both Meinecke's and Sachs and Georgi's reactions have been extensively tested in large series, and a summary of results in over 12,000 cases by various workers who compared the Sachs-Georgi with the Wassermann reaction in parallel series yielded a conformity percentage with the Wassermann of no less than 92.44 per cent. (Sachs and Georgi, 1920).

The most recent advance is that of Dreyer and Ward (1921), who follow in principle the method of Sachs and Georgi, but prefer as antigen, on the ground of greater stability, one recommended by Bordet and Ruelens (1919) for Wassermann tests. To this antigen, which, according to Bordet and Ruelens, can itself be flocculated in the presence of luetic serum, Dreyer and Ward add cholesterin. They claim that with a stable antigen and a refined technique

involving the quantitative expression of all degrees of flocculation, the test so conducted can lend itself to accurate standardization in terms of standard units.

Time and experience on the large scale will show how far this claim is justified and compatible with practicability in the hands of average workers, whose power to translate visual impressions into arithmetical language is notoriously variable. In treated cases particularly, the superiority of quantitative readings in flocculation tests over the purely qualitative evidence of the Wassermann would be warmly acknowledged, and it is to be hoped that the extensive parallel series which we understand are in contemplation may furnish important information on this point.

The above sketch is concerned solely with methods based on the mutual interaction of organic colloids and takes no account of the various and, on the whole, unsatisfactory tests which involve merely the precipitation of serum globulins by electrolytes.

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THE TOXIGENIC FEATURES OF STRAINS OF THE DIPHThERIA BACILLUS ISOLATED FROM HORSES AND FROM A MULE.

By G. F. PETRIE, M.D.

(From the Serum Department, Lister Institute.)

IN December, 1920, Capt. F. C. Minett, R.A.V.C., of the Royal Army Veterinary School, Aldershot, published an account of diphtheria bacilli isolated by him from eleven horses and one mule. He states that during the last year of the War and for some months after the Armistice the laboratory at the Army Veterinary School received for examination numerous specimens of pus derived chiefly from suspected cases of ulcerative lymphangitis; a condition in horses in which swelling of the lower parts of a limb or limbs is accompanied by abscess formation followed by ulceration. From this material he collected and investigated a number of diphtheroid strains including the bacillus of Preisz-Nocard and in the course of his inquiry discovered twelve strains of the diphtheria bacillus.

Nine of the strains were cultivated from lesions of the type named; the source of the remaining three is given below.

(1) A swab from a trephine opening in the cheek of a horse. A trephining operation had been practised prior to February, 1920, to relieve a nasal discharge. Although the discharge lessened and the animal remained in excellent condition the operation wound proved obstinate in healing and was still open on 17 October, 1920, when *B. diphtheriae* and streptococci were isolated from it. (Culture D 34 referred to below in the text and in the tables.)

(2) Pus from acneiform skin lesions. These consisted of a dozen superficial suppurating areas covered by scabs and mostly confined to the region of the withers; each was about the size of a threepenny bit.

(3) Pus from a contused fetlock. The lesion was not regarded as a lymphangitis.

Capt. Minett gives a detailed description of the characters of the twelve strains and states that five of them proved to be toxic; the remainder yielded no demonstrable toxin. He kindly sent six of the cultures to this laboratory; our thanks are due to him for the opportunity of examining them.

Particulars of the source and date of isolation of the six cultures forming the basis of the present communication are given in Table I; the symbols chosen by Capt. Minett to designate the cultures have been retained.

Microscopically the cultures are in no way distinguishable from the diphtheria bacillus. When grown for toxin production in Erlenmeyer flasks all give a typical surface film and in the case of "L" culture—the one mostly worked with—the "curtains" characteristic of a heavy, coherent, rapidly growing pellicle of the *B. diphtheriae* are in some batches exceptionally well developed.

Daily estimations of the hydrogen-ion concentration during the period of growth of a number of batches inoculated with cultures "L" and "D 34" were made; the resulting curves resembled those of standard toxin-producing strains of the *B. diphtheriae*.

All the strains formed acid from glucose and maltose but not from mannite and saccharose.

The data concerning toxin production are arranged in Tables II to VIII; and the results obtained on the first attempt at toxin production may be briefly summarized.

(1) Each of the six strains yielded a filtrate of which 0.1 c.c. killed a "250 gramme" guinea-pig within 48 hours when injected under the skin. Later tests showed that the M.L.D. of the various filtrates approximated to 1/100 c.c. more or less and that a mixture consisting of equal volumes of each gave an M.L.D. of 1/100 c.c. (Table II).

(2) 200 M.L.D.'s of the pooled filtrates when mixed with five units of diphtheria antitoxin and injected subcutaneously produced neither local nor general symptoms in a guinea-pig; a result demonstrating complete neutralization of the toxin by diphtheria antitoxin (Table IV).

(3) The L + dose of the several toxins from the six strains varied from 0.5 c.c. to 1 c.c. (Table V).

(4) Intracutaneous tests of the individual toxins gave skin reactions with amounts corresponding with the relation known to exist between the subcutaneous minimal lethal dose and the intracutaneous minimal reacting dose of diphtheria toxin (1 : 1/500) (Tables III and VI).

These results confirm Capt. Minett's conclusion that the cultures are veritable strains of the diphtheria bacillus. Recent experience indicates that two of them, namely "L" and "D 34," are equal from the point of view of toxigenic ability to the routine diphtheria strains—most of them derivatives of the No. 8 bacillus of Park and Williams—that are used in this laboratory. Thus, later batches of toxin made from strains "L" and "D 34" each gave an M.L.D. of 1/450 c.c. and an L + dose of 0.13 c.c. (Tables VII and VIII).

Cobbett (1900) reported an instance of horse diphtheria which in the following circumstances apparently conveyed the infection to a child: a little girl having fallen ill of diphtheria, Dr A. Mearns Fraser, M.O.H. of Portsmouth, while seeking the source of infection, found that a pony belonging to the child's father was ill with a purulent and sanguineous discharge from its nose. From the nasal mucus Dr Fraser isolated a bacillus morphologically indistinguishable from the diphtheria bacillus; Cobbett to whom the culture was sent proved that it was a true diphtheria bacillus.

Capt. Minett's findings strengthen the views put forward by Cobbett that horse diphtheria is of practical importance in relation to the Public Health and that the occurrence of the disease in horses throws light on the comparative frequency of "normal" antitoxin in their blood.

The discovery of the diphtheria bacillus in superficial septic lesions in the horse apart from specific infection of the nasal mucosa is paralleled by recent

observations on the human subject. Thus, there is growing evidence that in Man the *B. diphtheriae* is apt to be associated with a variety of chronic skin lesions or may become implanted upon cutaneous or subcutaneous lesions already infected with pyogenic bacteria, for example, war wounds and friction sores. Martin (1917) isolated virulent diphtheria bacilli from sores of this kind which were refractory to the usual methods of treatment. It is significant that they occurred in men of the Australian Light Horse. Dr Martin informs me that similar observations were made afterwards by others in Egypt and Palestine.

Capt. Minett's observations suggest that reciprocal contagion of horse and human diphtheria happens more frequently than has been hitherto suspected. Inquiries undertaken with this probability in mind when the circumstances of infection are obscure, may lead to the detection and prevention of cases of diphtheritic infection; and may indicate effective treatment with antitoxic serum.

Table I.

Designation, source and date of isolation of 6 out of 12 cultures obtained from septic lesions in horses by Capt. F. C. Minett, R.A.V.C.

Designation	Source	Date of isolation
L	Pus from suspected case of ulcerative lymphangitis in horse at A.	2. vi. 1919
G	" " " " " " " at M.H.	Prior to 4. vi. 1918
H	" " " " " " " at C.	6. ii. 1919
O	" " " " " " mule at L.	6. ix. 1919
D 16	" " " " " " horse at O.	28. x. 1919
D 34	Swab from trephine opening in cheek of horse at W.	17. x. 1920

Tables II, III, IV, V, VI.

Tests on guinea-pigs weighing *circa* 250 grammes with toxins derived from the 6 strains; cultures inoculated on 21. i. 21 and filtered on 28. i. 21.

Table II. *Subcutaneous M.L.D.*

Date of test	Culture	Dose	Day of death
4. ii. 1921	L	1/100 c.c.	3rd
"	G	"	8th
"	H	"	6th
"	O	"	9th
"	D 16	"	—
"	D 34	"	6th
10. ii. 1921	Mixture of equal volumes of 6 toxins	"	4th

NOTE. The animals that died from the subcutaneous inoculation of filtered cultures and of which the deaths are recorded in this and the following tables were examined postmortem and were found to present the appearances characteristic of diphtheria toxæmia in guinea-pigs.

Table III. *Intracutaneous test.*

Date of test	Culture	Dose	Cutaneous reaction on 4th day	Dose	Cutaneous reaction on 4th day
3. ii. 1921	L	1/25,000 c.c.	necrosis + +	1/50,000 c.c.	necrosis +
"	G	"	" + +	"	" tr
"	H	"	" + +	"	" + +
"	O	"	" +	"	" tr
"	D 16	"	" +	"	" tr
"	D 34	"	" +	"	" tr

NOTE. tr = trace: + = slight: + + = definite.

Table IV. *Neutralization of mixture of toxins of 6 strains by diphtheria antitoxin.*

Date of test	Dose	Result
10. ii. 1921	2 c.c. of mixed toxins = 200 M.L.D.'s + 5 units of diphtheria anti- toxin	No local reaction nor general symp- toms; progressive increase in weight

Table V. *Subcutaneous test of L + dose of toxins.*

Date of test	Culture	L + dose
14. ii. 1921	L	0.75 c.c.
19. ii. 1921	G	0.75 c.c.
"	H	0.75 c.c. nearly*
"	O	0.75 c.c. nearly†
17. ii. 1921	D 16	1.0 c.c.
21. ii. 1921	D 34	0.5 c.c.

* Death between 5th and 6th day.

† Death between 6th and 7th day.

Table VI. *Intracutaneous test of L + dose of toxin "L."*

Date of test	Culture	Dose	Result
9. ii. 1921	L	1/500 A.U. + 1/2000 c.c. toxin	0
"	"	" 1/1000 c.c. toxin	0
"	"	" 1/666 c.c. toxin	? tr necrosis
"	"	" 1/500 c.c. toxin	necrosis + +

NOTE. A.U. = antitoxin unit.

Tables VII and VIII.

Tests with later toxins derived from strains "L" and "D 34." "L" culture inoculated on 1. iii. 1921 and filtered on 10. iii. 1921; "D 34" culture inoculated on 2. iii. 1921 and filtered on 11. iii. 1921.

Table VII. *Subcutaneous M.L.D.*

Date of test	Culture	M.L.D. of toxin
21. iii. 1921	L	1/450 c.c.
"	D 34	1/450 c.c.

Table VIII. *Tests of L + dose.*

Date of test	Dose	Result with "L" toxin	Result with "D 34" toxin
2. iv. 1921	1 A.U. + 0.2 c.c. toxin	Death within 48 hours	Death within 48 hours
6. iv. 1921	" + 0.15 " "	" "	" "
9. iv. 1921	" + 0.13 " "	" "	Death on 4th day
4. iv. 1921	" + 0.10 " "	No local reaction: g.-pig gained weight	No local reaction: g.-pig gained weight

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COMPARATIVE IMMUNITY TESTS WITH SALINE VACCINES AND LIPOVACCINES.

J. PRATT-JOHNSON, M.B., B.S.LOND., D.P.H.Oxon.

From the Bacteriological Department, Lister Institute, London.

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THE presence of specific antibodies in the blood of immunised animals is of such general occurrence as to suggest that these substances are important factors in the mechanism of immunity. To what extent this is true is not known. In any case the antibody titre has been employed, perhaps too frequently, as a test of the immunity realised by the vaccination of man and of experimental laboratory animals. The type of immunity so arrived at may bear little or no relation to immunity as defined by ability of the vaccinated animals to withstand the dose or a multiple of the dose of the live organism or organismal protein which would otherwise kill the control unvaccinated animal.

The experiments here detailed were performed primarily for the purpose of studying the relative efficiency of lipovaccines and the ordinary saline vaccine suspensions. For this purpose it was planned to immunise various series of rabbits with vaccines prepared from a member of the *Salmonella* group possessing marked pathogenicity for the species (*Tenbroeck's Bacillus of Hog Cholera XII*), and, at a fixed interval after the close of immunisation, to test by the lethal dose method the degree of immunity, if any, attained. As we shall see, immunity as defined above was unfortunately not realised with any of the vaccines tried, and the experimental results here recorded bear simply on the lack of association of agglutinin development with genuine resistance to infection by the virulent live organism. In a subsequent paper (1921) some further and more successful attempts to obtain a solid immunity against the virulent strain in question are described.

PREPARATION OF LIPOVACCINES.

A lipovaccine may be defined as a suspension of killed bacteria in an oily vehicle. At first Le Moignic and Pinoy (1916) used mineral oils, which were subsequently replaced by vegetable oils such as olive oil, cotton-seed oil, almond oil, etc. In the French Navy lipovaccines have been employed on a large scale, and their preparation has been investigated by various workers, especially

Whitmore, Fennel and Petersen (1918), Olitsky (1918), and Rosenow and Osterberg (1919). It has been claimed that owing to the diminished toxicity of bacteria suspended in an oily vehicle lipovaccines may be administered in larger doses than saline vaccines, and that by virtue of their slower absorption a more lasting immunity is produced. The chief technical difficulty to be overcome in the preparation of lipovaccine is the removal of the watery envelope around the bacteria to be suspended. To this end various methods have been devised.

Method of Le Moignic.—The watery bacterial emulsion is centrifuged and the supernatant fluid removed. The semi-solid mass of bacteria is then again centrifuged and the remaining drops of watery fluid removed by means of a fine capillary pipette. A hydrophilic fluid of an oily nature, the composition of which is not published, is then allowed to remain in contact with the bacteria for some days, at the end of which time the bacteria, at first white, are penetrated by the oil and are stained reddish by the colouring matter in the oily fluid. The lipovaccine is sterilised by the combined action of "eugenol" and heating to 60° C. for thirty minutes.

Method of Whitmore, Fennel and Petersen.—The bacterial mass, after centrifugalisation, is dried *in vacuo* and finally broken up and incorporated in an oily vehicle by means of constant prolonged shaking in a special machine. The objections to this method are (1) the difficulty of breaking up the dried bacteria finely enough to obtain a uniform suspension, and (2) the question of ensuring the sterility of the vaccine, which is liable to contamination during the drying of the bacterial paste and its transference when dried to the special mixing machine. Further, it is doubtful if this method secures actual penetration of the oil into the bacteria.

Method of Olitsky.—After separation of the watery supernatant fluid by centrifugalisation, almond oil is added and a mixture made mechanically by vigorous shaking with glass beads. This method does not remove the water around the bacteria or secure penetration of the oil. The bacteria remain a greyish-white colour for many months and do not take up the colouring matter present in olive oil. Sterility is secured by heating to 60° C. for thirty minutes, the effectiveness of this low temperature in killing bacteria in an oily fluid demonstrating the presence of water in the lipovaccine.

Method of Rosenow and Osterberg.—The bacteria are collected by centrifugalisation and sterilised by heating or the addition of disinfectants. To the thick watery bacterial paste is then added cotton-seed oil containing 2 per cent. lanoline. The subsequent clearing process is hastened by the presence of the lanoline. The mixture is then heated under reduced pressure at 60° C., the contents being gently shaken until all bubbling has ceased. The water boils away, and the bacteria, which are at first of a greyish-white colour, become yellowish. Heating is continued until the opaque emulsion becomes almost clear.

An objection to this method is the employment of prolonged heating, which, if the brew be a large one, may extend up to three or four hours. The extent to which the antigenic value is affected would require investigation for each organism, and might be expected to vary also with the length of exposure to the heating process.

To ensure sterility of the final lipovaccine it is essential that a sterile bacterial emulsion be used in the first instance. Rosenow and Osterberg's method does secure penetration of the oil into the bacteria.

Eugenol method.—This process was suggested to the writer by a consideration of the analogous process of dehydration and clearing of pathological sections after staining by means of watery solutions. It will be remembered that the presence of mere traces of water produces clouding of the section owing to the incompatibility of water with xylol. Eugenol is a phenoloid substance present to a considerable proportion in commercial clove oil (about 80 per cent.), and has been adopted as an index of the purity of commercial clove oil. It behaves in every way similarly to clove oil but is preferable to it owing to the variable composition of the latter, which is liable to interfere with its clearing action especially when adulterated with alcohol.

The bacterial emulsion is first sterilised by heating to 60°C. for 30 minutes or 53°C. for 70 minutes. It is important to demonstrate its sterility because the subsequent process does not sterilise the thick emulsion. The emulsion is centrifugalised in a sterile tube plugged with cotton-wool and the supernatant fluid pipetted off. The deposit is again centrifugalised until it is packed down hard and the remaining drops of fluid removed with a sterile fine pipette. A few drops of absolute alcohol are then added to the centrifuge tube and the mass rubbed up with a small sterile glass pestle into a thick paste, which is gradually thinned down by adding more alcohol. The whole is again centrifuged and the alcohol removed by a fine pipette. A few drops of eugenol are then added and the sterile pestle again used to form a thick paste which is thinned down by the addition of more eugenol. In 15 to 20 minutes the bacteria are cleared up and penetrated by the eugenol. The lipovaccine is then made up by adding an estimated quantity of the eugenol suspension to sterile olive oil.

While this method has the advantage of simplicity and speedy preparation it is open to the possible objection that the alcohol washings are discarded. The presence of alcohol retards the subsequent clearing process by the eugenol, and also is liable to cause a precipitate when an alcohol-eugenol suspension is added to olive oil owing to the incompatibility of alcohol with olive oil. The antigenic value of the alcoholic washings which are discarded has not been investigated. The employment of alcohol is necessary to ensure the thorough breaking up and dehydration of the bacterial mass.

TECHNIQUE OF STERILITY TEST FOR LIPOVACCINES.

It has been shown that the sterilisation by means of heat of an oily suspension of living bacteria is an entirely different problem to the sterilisation of a watery suspension. The same temperature is required to destroy bacteria in oil as is necessary for bacteria in air. Under these circumstances in the preparation of lipovaccines it is essential that only dead bacteria be used for their preparation, as the high temperature necessary for the sterilisation of oils reduces or destroys the antigenic value of any suspended bacteria.

The addition of an oily fluid to a watery culture medium such as broth does not provide a satisfactory breaking up of the oil even by prolonged

shaking. In view of the importance of definitely ascertaining the sterility of lipovaccines it appeared advisable to work out a technique which would secure a more satisfactory breaking up of the lipovaccine in the culture medium. If equal volumes of 1 per cent. sodium carbonate solution and olive oil be shaken up and then added to a large volume of distilled water a uniform milky fluid is produced. If this fluid be examined under a low power of the microscope it will be seen that the oil is split up into fine globular particles which are suspended in the fluid. If now this picture be compared with a preparation of a few drops of olive oil shaken up in ordinary broth the oil appears as large globular masses, only a small proportion of the oil having been split up into fine globular particles. After numerous experiments the following technique was adopted for testing the sterility of lipovaccines :

0.5 c.c. lipovaccine is pipetted by means of a 5 c.c. pipette into a sterile test-tube containing 2 c.c. of 1 per cent. sodium carbonate solution, and the oil rapidly emulsified by bubbling air through the mixture by means of the pipette. The whole is then sucked up into the pipette and immediately transferred to a 250 c.c. flask containing 50 c.c. of broth and well shaken. The short period of exposure of the oil to the alkali does not appear to be a matter of any practical importance, as *B. typhosus* was found to be still living after an exposure of 45 minutes to 1 per cent. sodium carbonate. It was not found necessary to correct the reaction of the broth to allow for the addition of alkali for the organisms investigated, but this adjustment could be readily worked out to obtain any desired reaction of the final emulsified oil-broth. The preliminary emulsification in this way provides a more efficient sterility test for oily preparations.

COMPARATIVE TESTS OF AGGLUTININ-DEVELOPMENT IN RABBITS IMMUNISED WITH SALINE VACCINES AND LIPOVACCINES.

A 24-hour-growth of the bacillus of Hog Cholera XII was emulsified in saline, shaken, filtered and counted by the dark-ground method. The emulsion was heated at 53°C. for 80 minutes, cooled down and made up to contain 0.5 per cent. carbolic acid. The strength of the final sterile emulsion was 270,000 million per c.c. This stock emulsion was used for the preparation of saline vaccines and lipovaccines.

Saline vaccines.—From this emulsion two saline vaccines were made, viz. :

(a) 5000 million per c.c.

(b) 10,000 million per c.c.

by diluting with carbolic saline.

Rosenow lipovaccine.—25 c.c. of the above sterile emulsion was centrifuged, the supernatant fluid removed, and sterile beads and 20 c.c. sterile neutralised olive oil containing 2 per cent. lanoline added. The large centrifuge tube was placed in a water-bath at 60°C. and connected up with a water-pump, a reduced pressure of 70-73 mm. of mercury being maintained. During the heating the tube was shaken by hand, the clearing process being completed in 1½ hours ; 55 c.c. of sterile neutralised lanoline olive oil was added and the tube closed with a sterile rubber cork and the whole well shaken. After a satisfactory sterility test a lipovaccine was prepared for use of a strength of 15,000 million per c.c. by the addition of 5 c.c. of the emulsion to 25 c.c. lanoline-olive oil.

Eugenol lipovaccine.—4.4 c.c. of the sterile emulsion was placed in a sterile, plugged centrifuge tube. After centrifuging the supernatant fluid was removed by means of a pipette and the wet deposit again centrifuged, the last drops of fluid being removed by a fine pipette. The deposit was then rubbed up with a few drops of absolute alcohol into a thick paste by means of a sterile glass pestle, the paste being thinned down by the addition of further alcohol. After centrifuging, and pipetting off the alcohol, eugenol was added. At first a few drops were added and the mixture rubbed up into a paste by means of the glass pestle, the volume being subsequently made up to 2.4 c.c. After twenty minutes' treatment in eugenol a lipovaccine of 15,000 million per c.c. was prepared by dilution with sterile olive oil (0.93 c.c. added to 30 c.c. olive oil). The lipovaccine was found to be sterile.

Formalinised suspension.—A suspension was prepared from the original emulsion, and diluted down to a strength of 3000 million organisms per c.c. This suspension was sterilised by the addition of 0.1 per cent. formalin and was used throughout for all the agglutination tests.

Thirty-six rabbits were weighed and sorted out into three groups of similar weight distribution. Before inoculation the serum of all the animals was tested and found to produce no agglutination with the formalinised suspension except in one case where a weak reaction was obtained (1 in 40).

On November 2nd, 1920, the saline vaccine series received 5000 million organisms subcutaneously and the lipovaccine series (Rosenow vaccine 12 animals, eugenol vaccine 12 animals) 15,000 million organisms from their respective vaccines. On November 8th, 1920, the saline series received a further dose of 10,000 millions.

The temperature of all animals was taken night and morning up to November 11th, but except for a rise of 1–4° immediately following the first inoculation no special effects were observed. In no case was any marked local reaction obtained, and no induration or abscess occurred at the site of inoculation.

The serum of each of the thirty-six rabbits was tested on the ninth, thirteenth, sixteenth and twentieth days following the injection of the first dose of vaccine (November 2nd), and on each of these days, also, tests were made of the pooled sera of the twelve rabbits in each group.

The results of the pooled series are appended (Table I).

TABLE I.—*Agglutinin Titres of Rabbits after Inoculation with Saline Vaccines and Lipovaccines.*

Vaccine.	End-point titres on particular days after inoculation.			
	9th.	13th.	16th.	20th.
Saline	1280	2560	2560	2560
"Rosenow" lipovaccine	640	640	320	1280
"Eugenol" lipovaccine	80	160	160	320

I do not propose to detail the figures for the individual rabbits on the days in question, but it may be of interest to note the range of variation experienced in the three series (Table II).

TABLE II.—*Variation in Agglutinin Titre of Rabbits Inoculated with Saline Vaccines and Lipovaccines.*

	No. of animals yielding titres of							
	2560.	1280.	640.	320.	160.	80.	40.	20.
Saline vaccine	9	1	1	1	0	0	0	0
"Rosenow" lipovaccine	3	4	3	1	0	0	1	0
"Eugenol" lipovaccine	0	2	0	1	4	2	1	2

It will be noted that the saline vaccine yielded the best results, while the lipovaccines and especially the eugenol vaccine lagged far behind.

TEST FOR PROTECTION AGAINST THE LIVE ORGANISM.

On the twenty-second day (*i.e.* November 24th) 6 rabbits were taken from each group and given a test dose of 600 living organisms intravenously. The virulence of this organism for the rabbit had been tested previously on numerous occasions, deaths occurring within five to seven days with doses as low as 30 organisms. The actual minimal lethal dose was not ascertained, but from the preliminary experiments made it was clear that the test dose of 600 organisms represented not less than 20 minimal lethal doses. On the day of the test, however, 4 control animals were given 6000, 600, 600 and 60 organisms respectively. All died either on the fifth or on the sixth day.

The annexed table (Table III) shows the results with the vaccinated animals, none of whom survived the test dose. The presence of a high or a low agglutinin-content does not appear to have any influence on the date of death.

TABLE III.—*Date of Death of Infected Rabbits after Vaccination with Saline Vaccines and Lipovaccines.*

Saline vaccine.			Rosenow lipovaccine.			Eugenol lipovaccine.		
Rabbit.	Agglutinin titre.	Date of death.	Rabbit.	Agglutinin titre.	Date of death.	Rabbit.	Agglutinin titre.	Date of death.
No. 22 .	5,120 .	Nov. 28	No. 35 .	2,560 .	Nov. 28	No. 46 .	40 .	Nov. 30
„ 23 .	80 .	„ 29	„ 36 .	2,560 .	„ 29	„ 47 .	20 .	„ 29
„ 24 .	2,560 .	„ 29	„ 37 .	1,280 .	„ 29	„ 48 .	160 .	„ 29
„ 25 .	1,280 .	„ 29	„ 38 .	320 .	„ 29	„ 50 .	80 .	„ 28
„ 26 .	640 .	„ 29	„ 40 .	320 .	„ 28	„ 51 .	160 .	„ 28
„ 27 .	10,240 .	„ 29	„ 41 .	640 .	„ 28	„ 52 .	1,280 .	„ 29

As it was clear that no protection had been established, it was decided to reinoculate the remaining six animals of each series. On November 30th each received 15,000 million organisms from their respective vaccines, the effect of this second dose being to raise very considerably the agglutinin-titres of the animals receiving the lipovaccine, almost to limits equalling those of the saline vaccine series.

On December 12th a test dose of 210 live organisms was given to each intravenously. At the same time five control animals received respectively 210, 210, 105, 56 and 28 live organisms. All five died between the fourth and

seventh days, and as the annexed table (Table IV) shows, none of the vaccinated animals survived the test dose in spite of the high agglutinin-content of their sera.

TABLE IV.—*Date of Death of Infected Rabbits after Further Vaccination with Saline Vaccines and Lipovaccines.*

Saline vaccine.			Rosenow lipovaccine.			Eugenol lipovaccine.		
Rabbit.	Agglutinin titre.	Date of death.	Rabbit.	Agglutinin titre.	Date of death.	Rabbit.	Agglutinin titre.	Date of death.
No. 28 .	640 .	Dec. 15	No. 39 .	2,560 .	Dec. 15	No. 49 .	320 .	Dec. 15
„ 29 .	10,240 .	„ 16	„ 42 .	5,120 .	„ 16	„ 53 .	5,120 .	„ 14
„ 30 .	5,120 .	„ 15	„ 43 .	2,560 .	„ 14	„ 54 .	2,560 .	„ 15
„ 31 .	10,240 .	„ 15	„ 44 .	2,560 .	„ 14	„ 55 .	2,560 .	„ 15
„ 32 .	10,240 .	„ 15	„ 45 .	5,120 .	„ 15	„ 56 .	320 .	„ 15
„ 33 .	2,560 .	„ 15	„ 34* .	— .	—	„ 57 .	640 .	„ 15

* This animal died in the course of immunisation.

CONCLUSIONS.

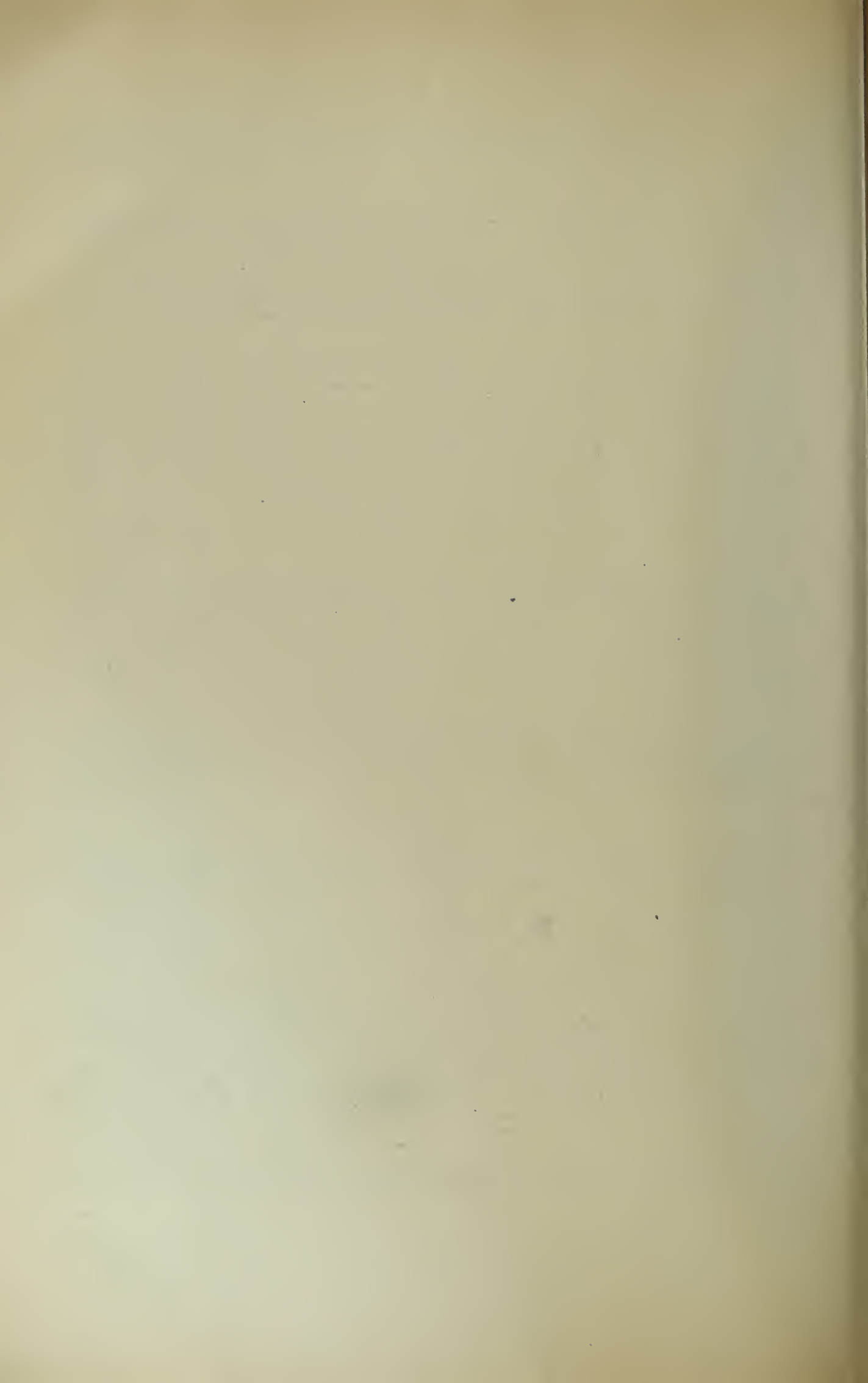
(1) Lipovaccines prepared and employed as above stimulate the production of agglutinins to a lesser degree than saline vaccines administered in the same doses.

(2) The presence of a high titre of agglutinins does not necessarily indicate the presence of an established immunity even against small doses of a highly virulent organism.

I here express my acknowledgments to the Governing Body of the Institute for facilities afforded me, and to Dr. Ledingham, Dr. Schütze and others for much helpful advice and criticism.

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FURTHER STUDIES ON THE PRODUCTION OF IMMUNITY IN RABBITS AGAINST AN ORGANISM OF HIGH VIRULENCE FOR THE SPECIES.

J. PRATT-JOHNSON, M.B., B.S.LOND., D.P.H.Oxon.

From the Bacteriological Department, Lister Institute, London.

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PREVIOUS attempts by the writer (1921) to protect rabbits against a virulent member of the *Salmonella* group (*B. cholerae suis* XII) by means of killed saline vaccines and lipovaccines having been unsuccessful, the following experiments were carried out with a view to testing the efficacy of immunisation with the live organisms of a related type of low virulence (*B. paratyphosus B*, type "Mutton"). No minimal lethal dose of the virulent organism had been determined, deaths having taken place with regularity after doses containing not more than 30 organisms. Tenbroeck (1918) has put on record certain experiments bearing on this question. He has shown that protection against lethal doses of "Hog cholera XII" can be secured by previous immunisation with live organisms of the "Mutton" but not of the "Schottmüller" type. It appeared therefore of prime importance to ascertain, if possible, the extent of this immunity in parallel experiment with the killed homologous and heterologous vaccines. The following vaccines were prepared:

- (1) Killed saline vaccine from *B. cholerae suis* XII.
- (2) Killed saline vaccine from *B. paratyphosus B*, type "Mutton."
- (3) Living vaccine of *B. paratyphosus B*, type "Mutton."

The living "Mutton" vaccines were prepared and counted on each day of inoculation.

Immunisation.—Six animals were employed for each series. Three injections were given at intervals of seven days, the first injection (March 23rd) subcutaneously, and the second (March 30th) and third (April 6th) intravenously. The doses of the killed vaccines on these three dates were 2000 millions, 2000 millions and 6000 millions respectively, while the three doses of the live "Mutton" vaccines were 30 millions, 30 millions and 300 millions.

Agglutinin-development.—On the thirty-fourth day after the first injection (*i. e.* April 26th) the sera of all the animals were tested against both "Hog cholera XII" and "Mutton" emulsions. Table I shows the agglutination results in end-point dilutions.

TABLE I.

Vaccine group.	Rabbit serial No.	"Hog cholera XII."	"Mutton."
Killed "Hog cholera XII"	81	800	0
	82	100	0
	83	400	0
	84	200	0
	85	400	0
	86	1600	0
Killed "Mutton"	87	0	1600
	88	0	1600
	89	0	800
	90	0	1600
	91	0	1600
	92	0	800
Living "Mutton"	93	50	6400
	94	50	3200
	95	200	6400
	96	50	6400
	97	200	6400
	98	50	6400

It will be noted that only those animals immunised with the live "Mutton" vaccine developed agglutinins for both organisms. The group effect in this case is, however, associated with a remarkably high agglutinin-titre for the homologous organism.

Protection tests.—On April 27th, the day following the agglutination tests above detailed, the inoculated animals received test doses intravenously of the live virulent "Hog cholera XII." Two animals of each series received 200 organisms, two received 1000 organisms, and the remaining two 1,000,000 organisms, while three control animals received respectively 100, 1000 and 1,000,000 organisms. The results are given in Table II.

TABLE II.

Test dose of living "Hog cholera XII."	Killed "hog cholera" vaccine.		Killed "Mutton" vaccine.		Live "Mutton" vaccine.		Controls.	
	Rabbit serial No.	Day of death.	Rabbit serial No.	Day of death.	Rabbit serial No.	Day of death.	Rabbit serial No.	Day of death.
200 organisms	81	May 4	87	May 2	93	<i>Survived</i>	101	May 2
	82	May 9	88	May 2	94	May 4	—	—
1000 organisms	83	May 3	89	<i>Survived</i>	95	<i>Survived</i>	102	May 2
	84	May 2	90	May 2	96	May 3	—	—
1,000,000 organisms	85	May 2	91	May 2	97	<i>Survived</i>	103	May 1
	86	May 2	92	May 2	98	May 4	—	—

It will be observed that of the six rabbits immunised with the killed "Hog cholera XII" none survived inoculation with the living culture, thus confirming previous results (1921).

Of the six animals immunised with the killed "Mutton" vaccine one survived, while of those which received the living "Mutton" organisms no less than three survived inoculation with living "Hog cholera XII." The series is a small one, and the immunity is not complete in any group, but the result demonstrates unequivocally the possibility of securing solid immunity against a highly virulent organism by vaccination with live members of a related type of low virulence. So far as this experiment teaches, no very obvious relation exists between survival, amount of test dose and date of death. With regard to antibody production, the chief point of interest is a possible association of immunity, not with the high agglutinin-titres following vaccination with the corresponding killed organism, but with the comparatively lowly titres which have developed in the serum as by-products of the action of a live avirulent but otherwise closely related organism.

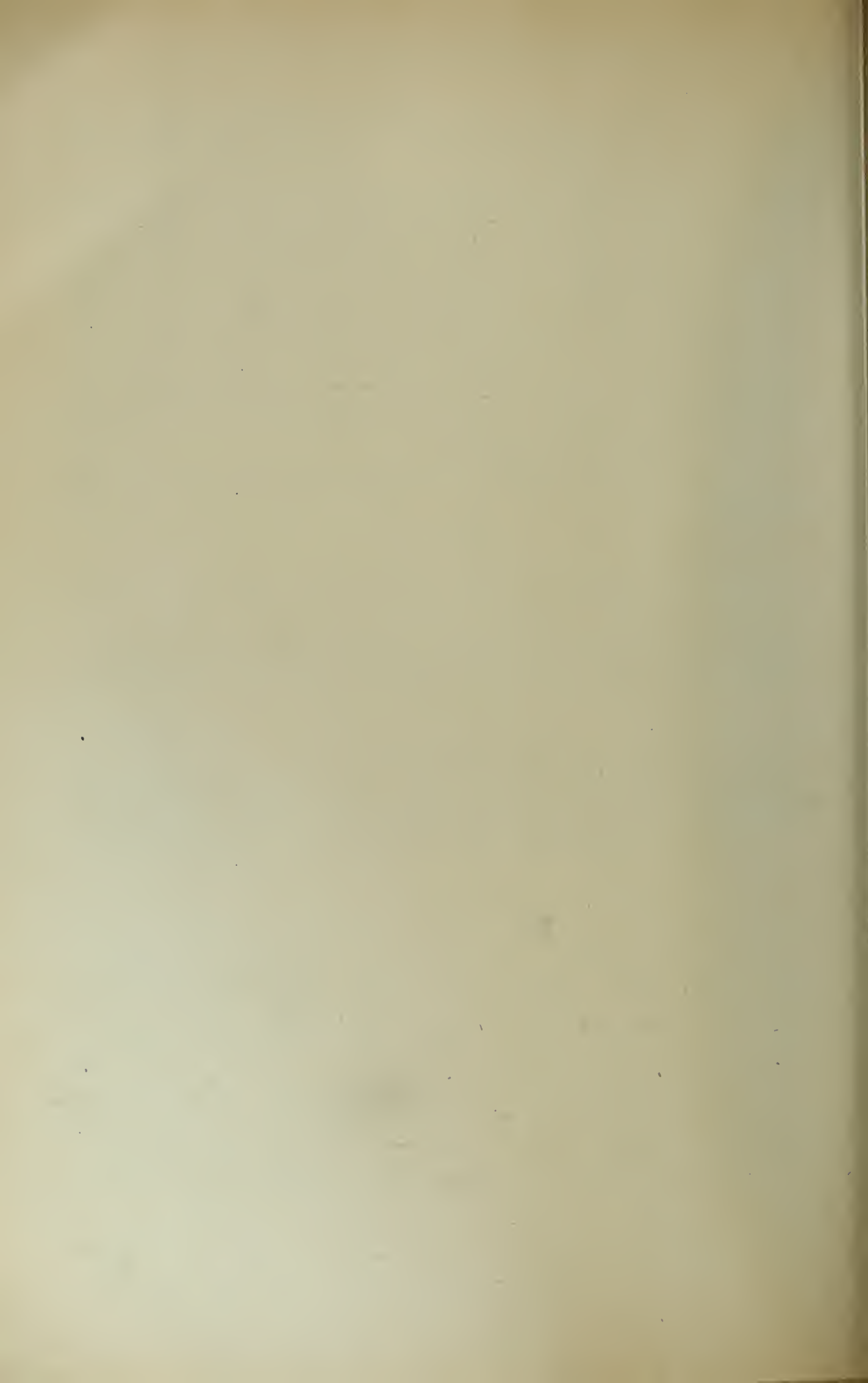
In all cases the cause of death was verified as due to infection with "Hog cholera XII" by the recovery of this organism in pure culture from the heart-blood.

CONCLUSION.

Experiments are described which demonstrate the possibility of securing the immunity of rabbits against an organism of high virulence by immunisation with a live related organism avirulent for the species. In this respect Tenbroeck's observations are confirmed, and it is further shown that killed vaccines prepared either from the homologous or from a related organism fail to give solid immunity against the live virulent strain.

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THE PERMANENCE OF THE SEROLOGICAL PARATYPHOID B TYPES, WITH OBSERVATIONS ON THE NON-SPECIFICITY OF AGGLUTINATION WITH "ROUGH" VARIANTS.

BY H. SCHÜTZE, M.D.

(From the Bacteriological Department, Lister Institute.)

(With 1 Figure.)

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1. INTRODUCTION.

IN a previous paper (Schütze, 1920), a serological classification of strains giving the *Salmonella* cultural reactions was described. By segregating those showing agglutinary relationship to one another, two groups were arrived at—the *P. enteritidis* Gärtner and the *B. paratyphosus* B. This latter includes types of diverse origin and pathogenicity, distinguishable in the laboratory by the absorption test; it embraces on the one hand the type labelled "Schottmüller," the well-known virus of paratyphoid B fever, on the other, with perhaps the least agglutinary affinity to it of any in the group, the more recently described "Hirschfeld" type (the *B. paratyphosus* C of that author). Between these extremes, with varying degrees of agglutinary relationship to the "Schottmüller" type, come organisms associated for the most part with food poisoning and animal epidemics, for example, the "Mutton" type and the "Hog Cholera" type. In no case can agglutination alone definitely decide to which type a particular strain belongs; only by absorption can certainty be obtained.

The degree of permanence possessed by serological bacterial types, in particular those demarcated by the finer distinctions of the absorption test, is not known.

Observations made upon the constancy of type manifested by a large number of strains during several years of laboratory cultivation are recorded

here, the species being *B. paratyphosus* B with its numerous absorption types. The results are of importance as indicative of the extent to which prototypes maintain their reliability as such.

2. "SUBSTRAIN" VARIANTS.

Criticism has been levelled at the subdivision of the paratyphoid B group by the absorption test on the ground that by choosing a different member of a serological type as prototype and working with serum derived from it, one arrives at a different arrangement of the strains within the types. But this does not appear to be the case, if notice be taken of the existence of what I have called "substrains." A substrain is one which contains less effective agglutinatorial antigen than another of the same type. A substrain will agglutinate with and absorb the agglutinins from the serum of a superstrain of the same type more or less badly according to the extent to which it is deficient in effective agglutinatorial antigen, but the superstrain always agglutinates to titre limit with and absorbs the agglutinins from the serum prepared from a substrain of the same type.

The most striking demonstration of a substrain is afforded by "Piper 1," a culture received from Capt. Fletcher and isolated by him from the urine of a paratyphoid case in 1917. Normal "Schottmüller" organisms were obtained simultaneously from the faeces. Recognised as atypical, the culture was sent to me for identification. On plating, the culture yielded two types of colonies, both giving the *Salmonella* sugar reactions; while one showed typical morphology, the other was of the kind now called "rough." With this latter, as it was a self agglutinator, little could be done. The former, "Piper 1," was found to agglutinate very poorly with and absorb not at all the Schottmüller serum

Table I.

Serum	Organism agglutinated	Titre						
"Tidy" serum: Unabsorbed	"Tidy"				1			
					12800			
"	"Piper 1"				1			
					400			
Absorbed with "Piper 1" (1 slope)	"Tidy"				1			
					12800			
"Piper 1" serum: Unabsorbed	"Piper 1"	$\frac{1}{100}$	$\frac{1}{200}$	$\frac{1}{400}$	$\frac{1}{800}$	$\frac{1}{1600}$	$\frac{1}{3200}$	Co
	"Tidy"	+++	+++	++	++	+	-	
"	"Tidy"	+++	+++	+++	++	+	-	
Absorbed with "Piper 1" ($\frac{1}{100}$ slope)	"Piper 1"	-	-	-	-	-	-	
	"	tr	-	-	-	-	-	
"	"	++	+	-	-	-	-	
"	"Tidy" ($\frac{1}{100}$ ")	-	-	-	-	-	-	
"	" ($\frac{1}{200}$ ")	+	-	-	-	-	-	
"	" ($\frac{1}{400}$ ")	++	++	+	-	-	-	

Absorption took place by adding the amounts of culture indicated to 1 c.c. of serum, diluted in the case of "Tidy" serum to $\frac{1}{100}$ and in the case of "Piper 1" serum to $\frac{1}{80}$, and incubating for 1 hour.

"Tidy" (see Table I). It could not on absorptive or even agglutinatorily grounds be regarded as of that type. As it conformed no better to any of the other paratyphoid B types, it was decided to carry out the so-called "mirror" test, *i.e.* to prepare a serum from the strain in question and absorb it with the various type strains. The rabbit yielded a serum with titre no higher than $\frac{1}{1600}$ and further inoculation failed to raise it. It will be seen from Table I that this lowness of titre was not due to any inagglutinability on the part of "Piper 1" itself; there was no better agglutination with the typical Schottmüller strain "Tidy." It will be seen too that absorption of the specific agglutinins is performed equally well by both "Piper 1" and "Tidy."

It would seem as if the antigen mosaic were defective in the case of "Piper 1," only a very small portion of that contained in a typical strain like "Tidy" being present or at any rate capable of engaging in the processes of agglutination, absorption and agglutinogenesis. The effective portion has, however, its counterpart in the more complete mosaic of the typical "Schottmüller" strain. "Piper 1" was therefore called a substrain of the "Schottmüller" type.

But further absorptions of "Piper 1" serum with the other paratyphoid B types proved that the classification of "Piper 1" was not so simple, for of the ten absorption types two besides "Schottmüller" were capable of absorbing the specific agglutinins from "Piper 1" serum, *viz.* the "Mutton" and the "Stanley" types.

Here, then, were three paratyphoid B types closely related agglutinatorily yet quite distinct absorptively, all of which completely absorb "Piper 1" serum. "Piper 1" is therefore a substrain to all three equally. The agglutinatorily antigen in "Piper 1" is apparently so limited in amount that it merely represents that antigen or a portion of that antigen which, in their more complex mosaics, "Schottmüller," "Mutton" and "Stanley" types have in common and by virtue of which they display their agglutinatorily relationship. "Piper 1" is, as it were, a common denominator of the three types in question.

Five other strains, resembling "Piper 1" in all respects, have been encountered, but none of them with a history indicating that it was isolated as such; one indeed ("Shanks") when received in 1916 was absorptively a normal "Schottmüller," when retested in 1919 it was seen to have degenerated into a substrain similar to "Piper 1." Two others, "Lister" and "Edinburgh," obtained from Prof. Stenhouse Williams and one old laboratory culture, "Wassermann," had no history, but presumably they had not been regarded as coinciding with paratyphoid B "Schottmüller," for they all three had "Supester" prefixed to their names. The fifth is described later. What clinical significance is betokened by the fact of an organism proving to be a substrain, it is impossible to say. It may be that some of the cultures isolated from time to time and termed inagglutinable paratyphoids are members of this antigenically very depleted group.

Not all substrains differ from the typical so markedly as these six. Simply

by making single cell cultures from a normal "Schottmüller," it was possible to separate out strains (Tidy A and Tidy B) that were in slight and varying degrees substrains to the original culture (Tidy).

Table II shows a series of absorptions demonstrating incompleteness when substrain acts on superserum and completeness when matters are reversed.

Table II.

Serum	Organism agglutinated	Titre							Con
		$\frac{1}{200}$	$\frac{1}{400}$	$\frac{1}{800}$	$\frac{1}{1600}$	$\frac{1}{3200}$	$\frac{1}{6400}$	$\frac{1}{12800}$	
“Tidy” serum:									
Unabsorbed	“Tidy”	+++	+++	+++	+++	+++	++	++	
Absorbed with “Tidy”	”	—	—	—	—	—	—	—	
” “Tidy A”	”	++	++	++	++	tr	—	—	
” “Tidy B”	”	++	++	++	++	tr	—	—	
“Tidy A” serum:									
Unabsorbed	“Tidy A”	+++	+++	+++	+++	++	+	—	
Absorbed with “Tidy”	”	—	—	—	—	—	—	—	
” “Tidy A”	”	—	—	—	—	—	—	—	
” “Tidy B”	”	++	++	+	tr	—	—	—	
“Tidy B” serum:									
Unabsorbed	“Tidy B”	+++	+++	+++	+++	+++	+	—	
Absorbed with “Tidy”	”	—	—	—	—	—	—	—	
” “Tidy A”	”	—	—	—	—	—	—	—	
” “Tidy B”	”	—	—	—	—	—	—	—	

Only the strain "Tidy" with complete antigenic mosaic can absorb from all three sera. The first substrain "Tidy A" can do so from its own and the serum beneath it in the scale, but fails to absorb from its superserum "Tidy," while the second substrain "Tidy B" cannot do so from either of its supersera "Tidy" or "Tidy A." The incompleteness of absorption is not so marked in these cases as it is where "Piper 1" is concerned nor is their antigen so reduced that the heterologous types "Mutton" and "Stanley" contain their counterparts and are thus capable of absorbing their sera. As both "Tidy A" and "Tidy B" can absorb "Piper 1" sera, though the reverse does not occur, the relationship which the strains and types bear to one another may be illustrated as in Fig. 1 (p. 334).

Another instance of prolonged cultivation producing a substrain is afforded by the single cell culture "Tidy A" which, together with the single cell culture "Tidy B," was, as a test of the permanence of their substrain characters, daily subcultured from broth to broth. At the end of about two months "Tidy A" no longer belonged to substrain I but, like the five other cultures already mentioned, to substrain III; it was also found to contain the "rough" variant. "Tidy B" subjected to about half that amount of subculturing had, however, undergone no change.

It has been observed that small changes of position in the scale of antigenic activity, as indicated by the absorption test, often occur. Only twice has a big alteration been recorded, in both cases a descent in the scale; "Shanks" with infrequent agar subculturing during the course of three years and the single cell culture "Tidy A" after 71 broth subculturings had both lost all

effective agglutinator antigen beyond what is found in members of sub-strain III.

With most variations from the normal, diagnosis is easily accomplished by the mirror test. When a culture has been debased to as low a level as that of substrain III in the diagram, it is not possible to allocate the culture to one particular type; *e.g.* "Piper 1" may equally well be a degraded strain of any one of the three types, "Schottmüller," "Mutton" or "Stanley." Another feature common to substrains is the difficulty experienced in making with them agglutinating sera the titres of which in any way equal those easily arrived at with full normal strains.

After intravenous inoculation with heat-killed saline emulsions (doses of 500 million, 2000 million and 6000 million with six to seven days interval)

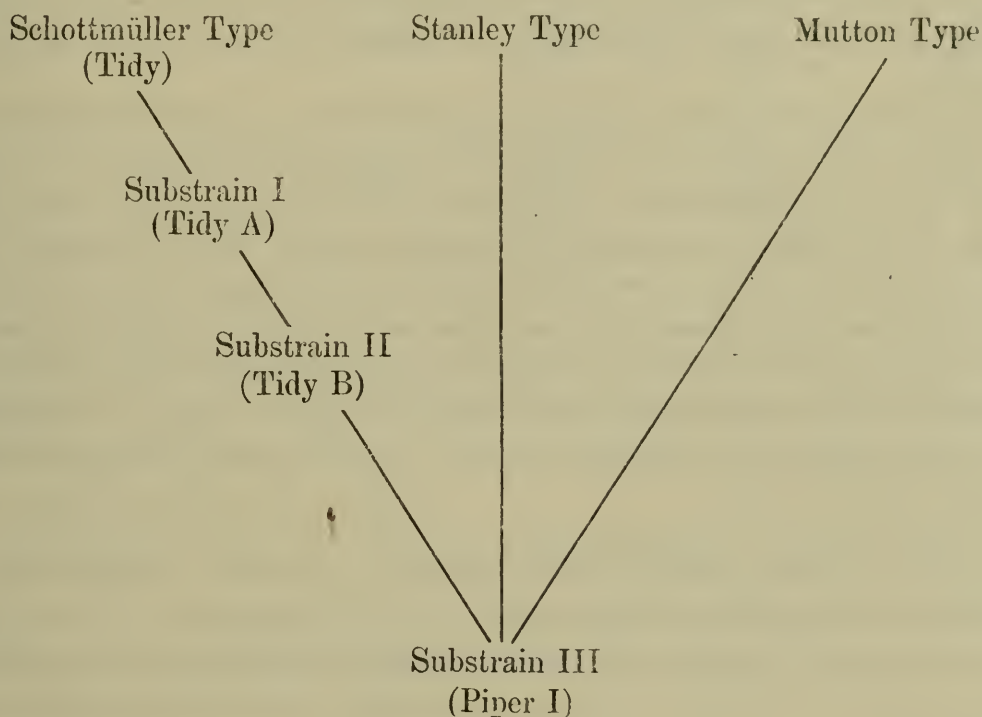


Fig. 1.

rabbits when they receive superstrains yield titres of $\frac{1}{6000}$ to $\frac{1}{12000}$, whereas when inoculated with substrains, they show titres of round about $\frac{1}{1600}$ and further and larger inoculations have little or no effect in raising the potency of the sera. The substrains are thus obviously less effective from an agglutinogenic point of view also.

In the estimation of the agglutinin content of a serum, such markedly substrain cultures as "Piper 1" are likely to make difficulties. In titrating a superstrain serum with a substrain emulsion, for example, one would naturally have to allow for its poor agglutinability, but if it is a serum prepared from a substrain similar to the one being measured, the substrain will register the titre to the full and the employment of any factor arrived at in work done with a superserum would lead one to erroneous results.

When the existence of these substrains is taken into account, the choosing of a different member of the same type as a prototype does not lead to an altered classification.

During the course of this work, it has been seen that even those strains whose serology was registered so long as six years ago, still maintain their places in the same absorptive types. Serologically no alteration except in "altitude" within the type has been recorded after laboratory cultivation extending over that period.

3. "ROUGH" VARIANTS.

There is one other variation that can and does take place during the conservation of cultures, and that is the one recently investigated by Arkwright (1921). The variant has been termed by him a "rough" and it differs from the parent form mainly in the morphology of its colonies, in its stability in saline emulsion, in its appearance in broth culture and in its serological character.

The variation from the normal in the case of the agar grown colonies may be anything from a mere occasional indentation in the edge of an otherwise normal colony to so marked a roughening and flattening of the surface that the growth resembles that of a spore-bearer on potato. In broth culture every stage is seen between a variant that sediments so completely as to leave an absolutely clear supernatant and one that shows but the slightest trace of abnormal precipitation at the bottom of a normally turbid broth culture.

Variations in colonial form and in saline stability do not go hand in hand. The variant giving the roughest colony is not necessarily the most saline unstable and vice versa.

4. NON-SPECIFIC AGGLUTINATION BETWEEN "ROUGH" ALIEN STRAINS.

The other variation already referred to, is a serological one. To a greater or less extent both agglutinary and absorptive relationship to the original culture is lost and, judged on these grounds, the variant could in some cases be regarded as a new type. The degree of alteration in serological character varies independently of the amount of colonial "roughness" and saline stability possessed by the variant. Although little or no agglutination may take place with a rough strain and its homologous smooth serum, the variant is not inagglutinable, it will respond well to a serum that has been prepared from a rough strain. And, what is very remarkable, is that the rough strains possess the power of agglutinating to a considerable extent with the sera of quite alien species when those sera have been made from rough strains. There exists a serological cosmopolitanism among rough cultures. Thus, for example, rough variants of "Gärtner," paratyphoid A and typhoid strains will agglutinate, sometimes to titre limit, with rough sera of the paratyphoid B group, while the smooth prototypes from which they have been derived, remain quite unaffected. Table III gives the titres of several smooth and rough strains for three rough alien sera.

Table III.

		Rough paratyphoid B sera		
Smooth and rough organisms of species alien to the agglutinating sera		"Schottmüller" type Titre = $\frac{1}{3200}$	"Hirschfeld" type Titre = $\frac{1}{3200}$	"Hog Cholera" type Titre = $\frac{1}{6400}$
Gärtner, "D. H. Bainbridge"	Smooth	$< \frac{1}{100}$	$< \frac{1}{100}$	$< \frac{1}{100}$
"	Rough	$\frac{1}{1600}$	$\frac{1}{3200}$	$\frac{1}{6400}$
Paratyphoid A, "S. O."	Smooth	$< \frac{1}{100}$	$< \frac{1}{100}$	$< \frac{1}{100}$
"	Rough	$\frac{1}{3200}$	$\frac{1}{3200}$	$\frac{1}{3200}$
Shiga, "550"	Smooth	$< \frac{1}{100}$	$< \frac{1}{100}$	$< \frac{1}{100}$
"	Rough	$\frac{1}{1600}$	$\frac{1}{1600}$	$\frac{1}{1600}$
Typhoid, "Howard"	Smooth	$< \frac{1}{100}$	$< \frac{1}{100}$	$< \frac{1}{100}$
" "Guy"	Rough	$\frac{1}{1600}$	$\frac{1}{400}$	$\frac{1}{800}$

It is seen that while the rough strains disclose an affinity for rough alien sera, the smooth strains fail to do so. That it was not a question of agglutination by serum *per se* whether immune or normal, was demonstrated by controls in which the rough strains remained unaffected. Indeed, for these rough strains serum has in certain concentrations an anti-agglutinator effect and this may be the reason for the frequency with which inhibition zones are met with when agglutinating rough strains.

It would seem then, as if there were genuine affinity between rough strains as such, but the relationship is not closer than that implied by the agglutination test. By absorption even the more closely related of the heterologous rough strains can be differentiated and the homologous ones identified, just as is possible with smooth strains.

5. THE SEROLOGICAL DIAGNOSIS OF "ROUGH" STRAINS.

In Table V are recorded absorptions carried out with a rough "Hog Cholera" serum. "Arkansas," like the strain labelled "Swine Fever," which, similar in every respect, was obtained from the Royal Veterinary College, is an old laboratory culture, and both have gone rough in the course of years of cultivation. To compare the identity of these cultures with more recently isolated and still smooth strains such as the "Hog Cholera XII" of Tenbroeck was impossible with the organisms in their respective states of roughness and smoothness. Table IV indicates what lack of reciprocity there is.

As it is apparently impossible to reconvert a rough into a smooth, Tenbroeck's culture was rendered rough by inoculating from broth to broth; after 7 days at 37° C. the second broth in the series showed signs of abnormal sedimentation and agar plating yielded the rough variant. Absorption of the

rough "Arkansas" serum was then quantitatively carried out with this rough "Hog Cholera" variant as well as with "Arkansas" itself and a rough variant

Table IV.

Serum	Organism agglutinated	Titre									
		$\frac{1}{200}$	$\frac{1}{400}$	$\frac{1}{800}$	$\frac{1}{1600}$	$\frac{1}{3200}$	$\frac{1}{6400}$	$\frac{1}{12800}$	Control		
"Arkansas" (rough) serum: Unabsorbed	"Arkansas" (rough)	+	+	+	+	+	+	+	+	-	-
"	"Hog Cholera XII" (smooth)	+	+	+	+	+	-	-	-	-	-
Absorbed with "Hog Cholera XII" (smooth)	"Arkansas" (rough)	+	+	+	+	+	+	+	+	-	-

Table V.

Serum	Organism agglutinated	Titre									
		$\frac{1}{200}$	$\frac{1}{400}$	$\frac{1}{800}$	$\frac{1}{1600}$	$\frac{1}{3200}$	$\frac{1}{6400}$	$\frac{1}{12800}$	Control		
"Arkansas" (rough) serum: Unabsorbed	"Arkansas" (rough)	+	+	+	+	+	+	+	+	-	-
Absorbed with "Arkansas," rough ($\frac{1}{2}$ slope)	"	-	-	-	-	-	-	-	-	-	-
"	" ($\frac{1}{4}$ slope)	tr	-	-	-	-	-	-	-	-	-
"	" ($\frac{1}{8}$ slope)	+	+	+	+	+	+	+	+	-	-
"Hog Cholera XII," rough variant ($\frac{1}{2}$ slope)	"	-	-	-	-	-	-	-	-	-	-
"	" ($\frac{1}{4}$ slope)	+	-	-	-	-	-	-	-	-	-
"	" ($\frac{1}{8}$ slope)	+	+	+	tr	+	+	+	+	-	-
"Hirschfeld," rough variant (1 slope)	"	+	+	+	+	+	+	+	+	-	-

of the so closely related "Hirschfeld" (paratyphoid C) type which, indeed, Tenbroeck (1920) considers to be serologically identical with "Hog Cholera." The rough "Hog Cholera" variant absorbs as well as the homologous

strain and it is therefore justifiable to regard "Arkansas" as a rough variant of the "Hog Cholera" type. The rough "Hirschfeld" variant, on the other hand, discloses a relationship to, but no identity with "Arkansas." And as the mirror test proved that it was not a substrain, a similar close relationship but lack of identity has been demonstrated between the rough variants of the "Hog Cholera" and "Hirschfeld" types, just as had previously been shown to exist between their smooth forms.

Many of the old standard laboratory cultures are found to have become rough during their years of conservation. Though a cultural diagnosis remains possible, their serological characters are obscure unless one takes into account the fact that they are variants from the normal. They are to be investigated, not so much by agglutination, which it has been seen, may be misleading, but by absorption, a test which apparently remains specific. The diagnosis of the rough strain "Arkansas," as here described, indicates in what manner the absorption test is to be performed. Given an unknown "rough" culture, one would prepare from it an agglutinating serum and then proceed to establish which of the type strains (in the form of their rough variants, of course) displayed affinity by completely absorbing the specific agglutinins from the serum.

6. THE DIAGNOSIS OF "ROUGH" STRAINS BY THEIR GROWTH INHIBITIONS.

There is one other method that may help in the typing of such rough strains and that is the investigation of their growth inhibitions. Attention has recently been recalled to this phenomenon in a paper by McLeod and Govenlock (1921). But here the old Eijkman (1904) procedure of direct inoculation of one strain upon another has been employed. By growing an organism in gelatine at 37° C. for 24 hours, cooling the culture to solidification and inoculating the sloped surface with a loopful of broth culture, one can determine what inhibitions to the growth of other organisms have been established. After one or two days at 22° C. if the inoculated bacillus is not inhibited, a roughening shows up along the track of the loop contrasting with the smooth surface of the gelatine and gradually developing into a definite line of growth.

A comparison of the mutual inhibitions and resistances of smooth and rough variants of the "Hirschfeld" and "Hog Cholera" types with those of the two rough strains "Arkansas" and "Swine Fever" gave the following results. Both "Hirschfeld" variants were capable of inhibiting the growth of both "Hog Cholera" variants as well as that of the two strains "Arkansas" and "Swine Fever," whose diagnosis is in question. On the other hand, neither the "Hog Cholera" variants, nor "Arkansas," nor "Swine Fever," though inhibition between themselves was complete, could inhibit either of the "Hirschfeld" variants. In this test also "Arkansas" and "Swine Fever" agree with the "Hog Cholera" rather than the "Hirschfeld" type. How far this method may be trusted to differentiate closely related serological types remains to be seen. Particularly will it be of convenience, if in this respect

substrains behave like the superstrains of their type and there is thus no necessity to prepare a special serum for the unknown strain in order to perform the mirror test. Laboratories would be relieved, too, of the need of preserving sera corresponding to the various types of this very large paratyphoid B group.

7. THE SEROLOGICAL PARATYPHOID B TYPES.

To the nine of these types, described in a previous paper, has subsequently been added a tenth, the "Abortus Equinus" type. This organism, the frequent cause of abortion in mares (Murray, 1919) is a *Salmonella* with marked agglutinary affinity to "Schottmüller" and its allied types, but absorptively quite distinct. The four most commonly encountered types remain: (1) "Schottmüller" (paratyphoid B fever), (2) "Mutton" (food poisoning cases and animal epidemics—it has been recovered from the rabbit, guinea-pig, mouse, calf, duck, parrot, pig, skunk, etc.), (3) "Hirschfeld" (paratyphoid C fever), (4) "Hog Cholera" (associated with the disease of that name); the remaining six have occurred sporadically in man and animals, in food poisonings and continued fevers.

8. CROSS-IMMUNITY WITHIN THE PARATYPHOID B GROUP.

The various types possess to a considerable degree the power of cross-immunisation as witnessed by the following Table VI. The rabbits were all immunised in the same fashion with 24 hours' living broth cultures of the various types—first inoculation 0.01 c.c. subcutaneously, second 0.01 c.c. and third 0.1 c.c. intravenously. After a lapse of 14 days the animals all received varying amounts of a 24 hours' broth culture of Tenbroeck's "Hog Cholera XII," an organism which is so lethal for rabbits that it kills when as few as

Table VI.

Rabbits immunised with living cultures of:	Immunity tested with living "Hog Cholera XII"					
	1 million \times M.L.D.		100,000 \times M.L.D.		10 \times M.L.D.	
	Survivals	Deaths	Survivals	Deaths	Survivals	Deaths
<i>Paratyphosus</i> B, "Schottmüller"	1*	0	0	6	0	3
„ "Hirschfeld"	1	0	2	0	1	0
„ "Mutton"	1	0	1	0	1	0
„ "Reading"	—	—	1	1	1	0
„ "Newport"	1*	0	—	—	—	—
„ "Stanley"	—	—	0	1	—	—
<i>B. enteritidis</i> , "Gärtner"	—	—	—	—	1	0
<i>B. coli</i> , "Escherich"	—	—	—	—	0	1
<i>Streptococcus faecalis</i>	—	—	—	—	0	1
<i>Paratyphosus</i> B, "Hog Cholera"†	—	—	—	—	0	2

* These two survivors were retested after a lapse of a year and still showed immunity to 10 and 100,000 \times M.L.D. respectively.

† Immunisation necessarily carried out with killed vaccine. A third rabbit succumbed to a single M.L.D.

50 to 100 single organisms are injected subcutaneously. The only exceptions to this scheme were the three "Hog Cholera" rabbits, which, because of the virulence of that type, were given the same doses killed by heating to 55° C. for half an hour, and the Streptococcus rabbit, which, being an animal immunised for other purposes, had received nine inoculations of killed streptococci (3000 million–15,000 million) followed by two of living streptococci, each 1000 million, all intravenous.

The results confirm Tenbroeck's statement (1918) that while the "Mutton" type (his "Swine Typhus" or animal paratyphoid B) protects against inoculation with "Hog Cholera," the "Schottmüller" type fails to do so. As far as the number of rabbits employed allows of a conclusion, other types besides "Mutton," including *B. enteritidis* Gärtner, also protect, but as has also been shown by Pratt Johnson in work published elsewhere, those rabbits which are immunised with *killed* cultures of the homologous "Hog Cholera" type, remain unprotected. In the reading of such results it must always be borne in mind that an apparently normal rabbit may have survived spontaneous infection by, say, the "Mutton" type and thus have acquired an unsuspected immunity against "Hog Cholera" inoculation, an immunity not only unsuspected but undemonstrable by serum examination, for the "Mutton" agglutination titre may have disappeared by the time the serum is tested. Both "Mutton" type paratyphoid B and *B. enteritidis* Gärtner are frequent invaders of animal houses and it is possible for the results of such immunity work to be confused.

9. *B. ENTERITIDIS* GÄRTNER AND *B. PARATYPHOSUS* B AGGLUTINATIONS.

As statements are made from time to time that these two organisms possess unstable serological characters and that a strain may at one time show those of a normal "Gärtner," and at another those of a normal "Mutton" or some intermediate stage between the two, it may be worth while noting here that not only have the laboratory conserved cultures of these two species constantly maintained their original characters, but in a number of isolations made during the investigation of spontaneously occurring epidemics in the animal house, no organism which could in any way be regarded as intermediate between the two, has been encountered. From animals dying spontaneously or in the course of experimentation (diet deficiencies or tubercle inoculations), some 116 "Gärtner" and 21 "Mutton" strains were isolated—111 of the former were recovered from rats and mice, 14 of the latter from guinea-pigs—and in every case the nature of the organism was unequivocally declared without any indication of a confusing serological cross-relationship.

SUMMARY.

1. Among a large number of strains belonging to the various absorption types of the paratyphoid B group and kept under observation in laboratory culture over a number of years, constancy of type has been demonstrated.

2. Only two alterations in the serological nature of certain cultures have been noted; they were due (a) to the development of so-called "rough" variants, (b) to the degeneration of strains into antigenically less effective "substrains."

Though both are variations within the limits of the absorption type, the serological character of the affected strains may be so obscured that a greater variation than has actually taken place may be ascribed to them, unless the precautions indicated are observed.

3. When "rough" strains are in question, agglutination results are to be mistrusted, for a marked cosmopolitanism in respect of this test has been seen to exist among such alien species as *B. enteritidis* Gärtner, *B. paratyphosus* A and *B. paratyphosus* B.

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No 19

THE ASSOCIATION OF THE VIRUS OF TYPHUS FEVER WITH THE VARIOUS BLOOD ELEMENTS.

J. SÉGAL, M.D., L.Sc.PARIS, D.T.M.LOND.

From the Bacteriological Department, Lister Institute, London.

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As the virus of typhus fever has not as yet been definitely cultivated, one is obliged to make use of virus-containing material (*e. g.* blood and brain) from infected animals in all experimental investigations on this disease.

This is a great drawback, as much unnecessary protein (tissue cells, etc.) is added to the volume of the inoculum.

The desirability of having the virus, especially for immunisation purposes, in as pure and as concentrated a form as possible is obvious, and it was with this object that the following experimental work was carried out.

ASSOCIATION OF THE VIRUS WITH THE LEUCOCYTES.

In 1912 Charles Nicolle brought forward experimental evidence to show that the leucocytes form the principal, if not the only infective or virus-carrying portion of the blood in typhus fever, the virus being apparently contained within these cells.

The observations supporting this assertion were as follows:

In a severe case of typhus fever, blood was taken on the seventh day of disease and distributed in sterile centrifuge tubes containing 2 per cent. citrate solution (18 c.c. blood to 2 c.c. citrate solution). Some of this blood was centrifuged during twelve minutes (speed not indicated) and the supernatant citrated plasma pipetted off and kept. The leucocytic layer of the deposit was then carefully removed and emulsified in saline. Similarly a few c.c. of red cells were collected from the deepest layer of the deposit and suspended in saline. These suspensions were again centrifuged during ten minutes and the supernatant fluid removed. Monkeys (*M. sinicus*) were then inoculated with—

- (1) 5.0 c.c. of the citrated plasma.
- (2) About 1 c.mm. of the leucocyte layer.

This material on microscopical examination was found to contain more red cells than leucocytes, the latter amounting at most to one-fourth or one-fifth of the total number of cells.

- (3) 2.5 c.c. of washed red cells.
- (4) 5.0 c.c. of whole citrated blood.

The result of this experiment was as follows :

The monkey inoculated with whole citrated blood developed a typhus infection of medium severity after an incubation period of twelve days, whilst the animal which received the washed red cells had an abortive attack of five days' duration. On the other hand the inoculation of the cells from the leucocyte layer gave rise to a severe infection after a short incubation period of six days.

A second experiment was performed in a precisely similar manner with the blood taken on the thirteenth day of a severe case of typhus fever.

On this occasion the separation of the leucocytes from the other blood cells had been more successful, only a small proportion of red cells being present.

The results obtained were very similar to those of the first experiment. The inoculation of 5 c.c. citrated plasma produced a mild infection lasting seven days, and no reaction at all occurred in the monkey receiving the 3 c.c. washed erythrocytes. The animal inoculated, however, with the 1.0 c.mm. of leucocytes developed after seven days' incubation a very severe infection.

From these experiments Nicolle has concluded that the virus of typhus fever present in the circulation is contained within the leucocytes, the virulence of the plasma being due to the *débris* of leucocytes that it might contain.

This assertion has met with almost general acceptance, and is quoted in many text-books as an established fact (Dopter and Sacquépée, Kolle and Hetsch, etc.). The literature, however, contains certain criticisms of Nicolle's work. Kusama (1920), who also quotes Miyajima, contends, and not without reason, that the leucocyte material in these experiments would contain blood-platelets as well as leucocytes. They also point out that the possibility of the virus being free in the blood and of being brought down with the leucocytes and platelets by virtue of a similar specific gravity has not been disposed of. Da Rocha-Lima (1919) does not admit the validity of this latter objection, for, in his opinion, centrifugalisation during twelve minutes would not be sufficient to throw down an invisible virus. He argues, however, that 3.0 c.c. of red cells should certainly contain as many leucocytes as would be present in the 1 c.mm. of white cells in Nicolle's experiments, and should therefore have produced infection. It is quite obvious that only the complete separation of the leucocytes from the other blood-elements will be able to answer this question definitely.

ATTEMPTS TO CONFIRM NICOLLE'S ASSERTION BY THE PRODUCTION OF A CONSIDERABLE EXTRAVASATION OF LEUCOCYTES.

By the separation of the leucocytes from the other blood elements a concentration of the virus should readily be realised, but it is impossible to obtain leucocytes from the blood in any quantity, and exceedingly difficult to separate them from the other blood-cells. This difficulty was overcome by making use of the white cells which appear in the peritoneal exudate after injection of sterile broth.

Typhus-infected guinea-pigs at different periods after infection received 10 c.c. of sterile broth intraperitoneally. This injection was practised slowly in order to avoid traumatism of the capillaries and extravasation of blood. These animals were killed after intervals of from two to five hours. The abdominal wall was carefully dissected out and the exudate sucked up by means of a Pasteur pipette through a small opening made in the peritoneum. In most cases it was found that the cells present in the exudate consisted entirely of leucocytes (principally polymorphonuclears). In certain instances, however,

the fluid had a uniform rose tint, which was shown by microscopical examination to be due to the presence of numerous red cells.

It might possibly be argued as an objection to this method that the leucocytes contained in the peritoneal exudate will not necessarily carry the virus. This objection is a perfectly valid one, but we have, of course, to consider the time which has elapsed between the injection of the broth and the withdrawal of the exudate. These leucocytes come from the blood, passing out of the local capillaries in response to the presence of the broth in the peritoneal cavity. The first of these cells to appear in the exudate will therefore be those present in the blood-stream at the moment of the inoculation. These leucocytes should be virus-carrying ones. Later, as a result of the drain on the leucocytes of the circulating blood, those in the bone-marrow may be mobilised to make good the deficiency. Some of these in all possibility would appear in the exudate after a very short stay in the circulation and might not carry with them the typhus virus.

The proportion of these non-infective leucocytes in the peritoneal exudate would increase with the time that had been allowed to lapse between the inoculation of the broth and the withdrawal of the contents of the peritoneal cavity. However, it seems fairly safe to presume that the fluid withdrawn from the peritoneal cavity in the early stages of the formation of the exudate, two to five hours after the inoculation, would be composed, at any rate in greater part, of leucocytes present in the circulation at the moment of its inception. It should therefore contain a large proportion of virus-carrying cells.

Guinea-pigs were inoculated intraperitoneally with 4 to 5 c.c. of this exudate (containing in certain experiments over 58,000 leucocytes per c.mm.) or with the leucocytes obtained by centrifugalisation of a similar volume of fluid and emulsified in saline. In one experiment the leucocytes were extracted with distilled water.

Results.—In no case (four experiments) did the inoculation of leucocytes so obtained produce infection.

Similar negative results were obtained with the whole exudate when it contained leucocytes only (three experiments), whereas infection ensued in the animals inoculated with a blood-containing exudate (two experiments).

These experiments were controlled by the simultaneous inoculation of the blood or brain of the experimental animals, showing them to be infective in every case.

The guinea-pigs that did not react to the leucocytes or whole exudate inoculation were later tested for immunity.

The following temperature charts are typical of the results obtained in the above experiments.

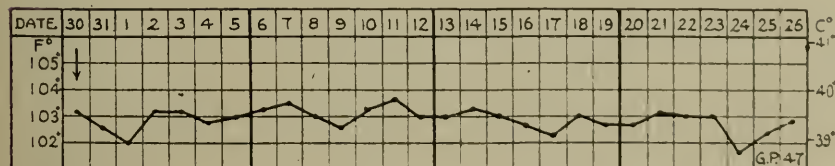


CHART 1.—Guinea-pig 47 inoculated with leucocytes obtained from 5 c.c. exudate and emulsified in saline.

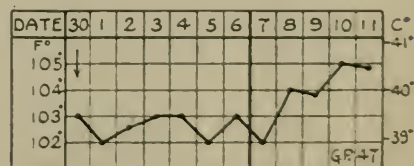


CHART 1A.—Guinea-pig 47 tested for immunity.

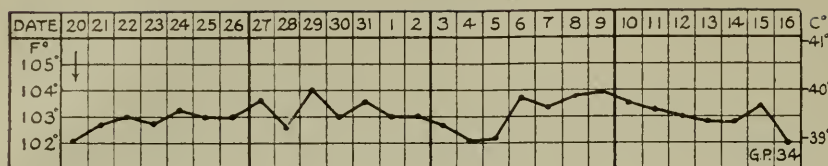


CHART 2.—Guinea-pig 34 inoculated with leucocytes of 4 c.c. exudate extracted with distilled water.

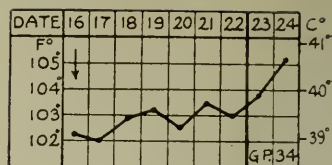


CHART 2A.—Guinea-pig 34 tested for immunity.

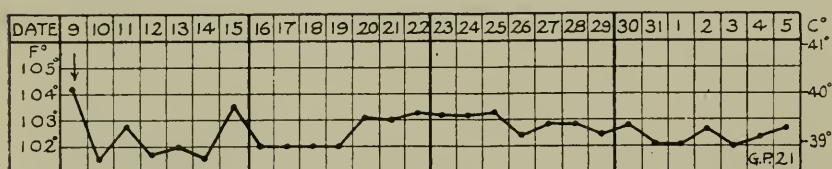


CHART 3.—Guinea-pig 21 inoculated with 4 c.c. whole exudate.

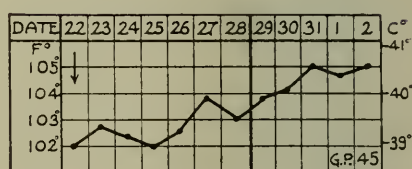


CHART 4.—Guinea-pig 45 inoculated with 5 c.c. of a pinkish exudate (containing about 30 per cent. of red cells).

ASSOCIATION OF THE VIRUS WITH THE PLATELETS.

At this juncture my attention was drawn to the work of Kusama (1920), who claims that there is an intimate connection between the virus of typhus and the blood plates.

This statement is based upon three experiments on Japanese monkeys. The inoculation of a quantity of platelets (free from leucocytes) obtained from 2.5 c.c. blood produced infection in the three cases, whereas the injection of the plasma obtained from a similar volume of blood and entirely freed from cells invariably gave negative results.

Experiments on guinea-pigs carried out on similar lines have given complete confirmation of the findings of this investigator.

The blood was received into an equal volume of a 2 per cent. citrate solution in saline containing 1 per cent. glucose (about 10 c.c. solution for each guinea-pig), centrifugalised 5 to 7 minutes at about 3000 revolutions per minute and then allowed to stand overnight in the cold room. The supernatant plasma, having a milky appearance, was then carefully removed and centrifugalised for forty-five minutes at about 6000 revolutions per minute. It is then found by microscopical examination that the yellowish supernatant fluid is cell free and the white sediment consists entirely of blood-plates.

Results.—Plasma carefully freed from cells by prolonged centrifugalisation (four experiments) proved non-infective even in large doses (5 c.c.), whereas the inoculation of platelets (six experiments) invariably produced infection even with so small a quantity as that obtained from 1.5 c.c. blood.

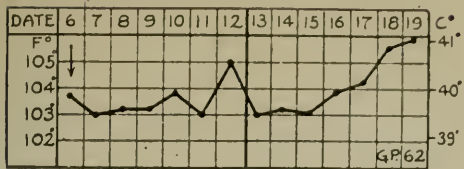


CHART 5.—Guinea-pig 62 inoculated with platelets from 4 c.c. blood. The cultures of the blood and brain of this animal were found to be sterile. Passed to Guinea-pigs 81, 82 and 83 with positive results.

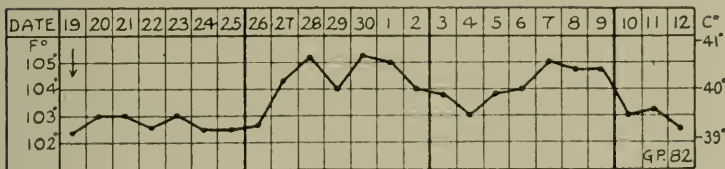


CHART 6.—Guinea-pig 82 inoculated with brain of Guinea-pig 62.

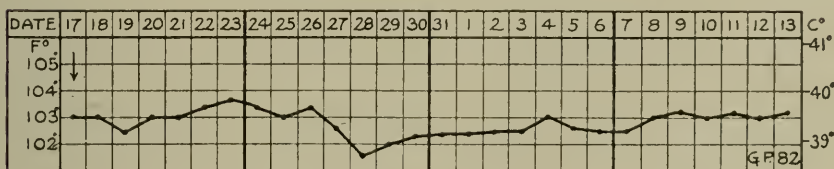


CHART 6A.—Guinea-pig 82 tested for immunity.

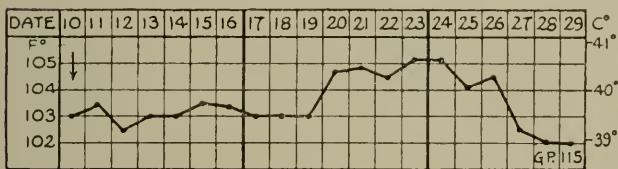


CHART 7.—Guinea-pig 115 inoculated with platelets from 1.5 c.c. blood.

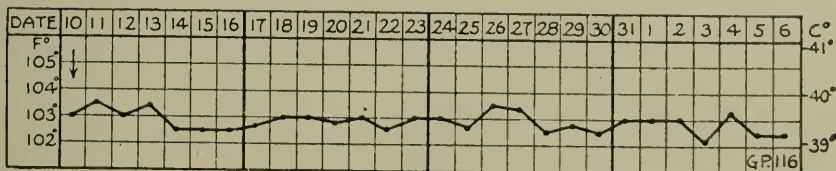


CHART 8.—Guinea-pig 116 inoculated with 5 c.c. plasma.

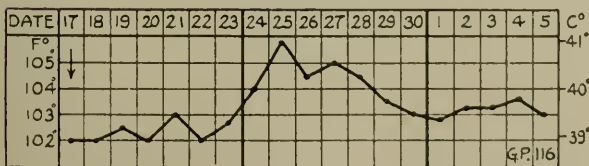


CHART 8A.—Guinea-pig 116 tested for immunity.

The leucocyte layer of centrifugalised citrated blood can be shown to contain an abundance of platelets even when the blood has only been centrifugalised during five minutes at a moderate speed. This fact may explain the findings of Nicolle, who, it will be remembered, worked with the leucocytic layer of blood which had been centrifugalised a much longer time and must therefore have contained platelets in still greater quantity.

A high degree of concentration of the virus can thus be realised by the separation of the platelets by fractional centrifugalisation from large quantities of blood. With such a concentrated virus it has been found possible to infect guinea-pigs by the inoculation of a small volume in the anterior chamber of the eye and by intratesticular inoculation. In this latter case a marked atrophy of this organ has followed, which did not occur in the control animals inoculated with the same volume of platelets obtained from normal blood. It has also facilitated the experimental infection of lice in joint work with A. W. Bacot (Bacot and Ségal, 1922).

A field of experimental vaccination is also opened up and work along these lines has already commenced. Although it is too early to draw any conclusions, the result of the first experiment on guinea-pigs is encouraging.

CONCLUSIONS.

(1) Leucocytes obtained from peritoneal exudates of typhus-infected guinea-pigs do not carry the virus.

(2) The virus seems to be intimately connected with the platelets.

(3) By the separation of these elements from a large quantity of blood a high concentration of virus can be realised for experimental purposes.

To many members of the staff of this Institute I am indebted for valuable help and advice, and I am especially grateful to Dr. Bedson for assistance in the preparation of this paper.

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No 90

AN INTRODUCTION TO THE STUDY OF HÆMATOPHAGY.

By H. M. WOODCOCK, D.Sc.LOND.

Fellow of University College.

(Head of the University of London Department of Protozoology.)

Protozoologist to the Egyptian Expeditionary Force.

I.—HÆMATOPHAGY AS A NORMAL OCCURRENCE: THE NATURE AND ORIGIN OF BLOOD-PLATELETS AND OF THE KURLOFF-BODIES.

INTRODUCTORY.

I PROPOSE to call by the name of *hæmatophagy* the function which may be exercised by a cell of ingesting blood-cells and elements of various kinds; a cell behaving thus may be termed a *hæmatophage*; and so on. The words phagocytosis, phagocyte, etc., have become inseparably associated with the idea, merely, of the removal and destruction of some effete cell or deleterious microbe. Little or no attention has been paid hitherto to the effect or result of this process upon the devouring cell. But in regard to the ingestion of blood-cells, the question of the use made of this "solid" nutriment is so important that I do not consider the term phagocytosis, with its conventional, limited significance, is any longer suitable. The terms here proposed are to be associated with the idea of a particular kind of food taken up by the cell, and the extent to which this nutriment can be digested and utilized by the latter. My object in this paper is to indicate some of the results of the exercise, normally or in pathological conditions, by different types of cell and with different consequences, of this function of hæmatophagy.

It is very much to be regretted that, owing to the enormous expense incurred at the present day in the production of coloured plates, it is impossible to have any to illustrate my work. The illustrations suffer,

unavoidably, in being in black and white only. I am deeply indebted to Miss Rhodes for the valuable assistance she has given me; not only in making the original coloured drawings, which have been submitted for the Editor's inspection, but also in redrawing a selection of them in black and white, from which the figures here given are reproduced. I am also greatly obliged to Dr. D. J. Reid, for his kindness in taking the excellent photomicrographs. Lastly, I desire to express my grateful thanks to my friends, Dr. J. D. Thomson and Dr. Ledingham and to Professor Boycott, for their most helpful advice and instructive criticism; whatever merit the paper may have would be distinctly less but for their kind assistance. But I ought, perhaps, to add that my wish to thank these gentlemen is not to be taken as necessarily meaning that they agree with my conclusions.

Considerations of space prevent any detailed discussion of the enormous quantity of literature relating to the various subjects dealt with. I have to confine myself, therefore, to the mention of only very few papers, which are of importance in relation to the views here adopted.

TECHNIQUE.

General Remarks.—The work has been entirely a cytological study, and in such the question of technique is, of course, of much importance. Far more important, however, is the interpretation of the appearances obtained; from the nature of the case, the right view or conclusion is a matter entirely of correct interpretation. And for a correct interpretation, a thorough knowledge, not only of the reliability but of the vagaries and idiosyncrasies of any particular stain employed is essential. This has certainly not been sufficiently realized in connexion with what is undoubtedly, on the whole, the most valuable staining-method for blood-elements which we possess. I refer, of course, to all those stains which may be comprehensively included in the category of Romanowsky stains—the red-blue combinations.

There are especially two points which have to be borne in mind in deciding the significance of any bodies of unknown or enigmatical character from their appearance when so stained. The first is the tendency to enormously overload with stain any cell-organ or inclusion for which some component of the stain has a strong affinity. Other stains—of “regressive” character—may also behave thus, but in their case the excess of the stain can be extracted by appropriate differentiation; in the case of Romanowsky combinations, differentiation to what would be the right extent is rarely feasible because of the liability of a particular colour (usually the red) to disappear. Thus it happens that the most minute granules are often rendered so conspicuous that they assume an air of importance which in reality they are far from possessing.

The second point is that, because a granule or a clump of granules stains red (or purplish-red) by this method, it does *not* follow that these

granules consist of chromatin and represent nuclei or true, functioning nuclear material. Their chromatinic nature must be tested by means of one of the well-known standard nuclear stains. As a protozoologist, I am astounded when I consider the quantity of work which has been published, often by competent investigators, in which such *red-staining* granules have been, *on that account* regarded as indicating the nucleus or the nuclear material of some parasite; and in which, moreover, a superstructure of a complicated life-cycle, with gemmules, gametes, spores and all manner of phases, has been built up on that fragile foundation alone. A remarkable feature is that these parasites, with their complex life-histories, are nearly always regarded as Protozoa, no account being taken of the absence, in many cases, of cytoplasm, or of any indication that these various bodies show the structure of a differentiated cell, characteristic of all Protozoa.

Two examples illustrating the above points may be given which will be within the cognizance of, or can readily be observed by anyone interested in blood-work. In an ordinary dried blood-smear, stained by Giemsa, let us say, the nuclei of the leucocytes present a beautiful picture, varying in hue (according to their type), from rich purple-red to almost brick-red. The whole nucleus is deeply stained, but rather darker masses of varying size and shape can usually be distinguished. Now this appearance of a huge red-staining nucleus by no means corresponds with the appearance of the actual chromatin present, as can be seen by a comparison of a wet-fixed film, stained, say, by iron-hæmatoxylin and differentiated in the usual manner. In such a film, the nucleus of a leucocyte does not occupy, relatively, so much space in the cell, and comparatively little of it is stained black, or greyish-black, indicating the actual amount of chromatin present. The grains of chromatin are disposed chiefly around the nuclear membrane, but a few occur also on the strands of the reticular network. All the ground-substance has practically the same tint as that of the cytoplasm, according to whatever counter-stain has been used; if none, it is neutral to greyish. (For figures illustrating the comparative appearances of the nuclei of the leucocytes of birds, when stained by these contrasting methods, see Woodcock [16].) A very large proportion, therefore, of the red-staining nuclear mass, in a Giemsa-smear, is not chromatin at all, but merely nuclear ground-substance.

The second example, on which I wish to lay particular emphasis, is the known variation in the staining character of the red corpuscles, when altered in any way. Thus, a ruptured, flattened corpuscle is often stained a strong red colour. Further, corpuscles are seen occasionally, probably effete or breaking-down, in or upon which the stain has been deposited in the form of red dots; manifestly certain fine granules are present in such cases which have a marked affinity for the red component of the stain (I am not referring, of course, to punctate basophilia). Again, under the influence of certain of the malarial parasites, red-staining granulations are regularly formed in the infected corpuscles. These vary in tint according to the type of parasite. Maurer's dots in corpuscles infected with the malignant tertian parasite, are of a much stronger red and larger, more blotchy in character than are Schüffner's dots in the case of benign tertian. *The important point to recognize is that certain products of the alteration of the substance of red blood-corpuscles may stain red by Romanowsky stains.*

It is seen, therefore, that, on the one hand, different bodies or structures, even though very closely related, may stain somewhat differently, in their normal phase and under constant conditions; and on this account they can be easily distinguished. And, on the other hand, the same organella or material may stain differently according as any slight alteration occurs in its composition; and this difference in staining character may give the object an entirely different appearance and render its recognition difficult. Hence the interpretation of the results of Giemsa-staining may appear to be most paradoxical; nevertheless, herein lies the secret both of the success and of the pitfalls attending its use.

The Giemsa-stain is invaluable for blood-work; but it *must be controlled* by a known, reliable, chromatinic stain such as hæmatoxylin—any of the various modifications will serve. Without the use of *both* these types of staining-method, I could not have gained enlightenment upon this subject of hæmatophagy.

Methods.—I have used human blood, and the blood and hæmatopoietic organs of the guinea-pig and kitten. Most of the work has been done upon the guinea-pig. Except where otherwise stated, all the preparations are from normal animals.

Most of my permanent preparations have been made according to one of the two following methods: (*a*) Smears: These were rapidly dried in air, fixed with absolute alcohol and stained with Giemsa for half an hour, the strength of the solution used being three drops to the cubic centimetre. (*b*) Films: These were made on a cover-slip and were fixed wet, i.e., by dropping them face downwards on to the fixative. As fixative, I used a special mixture of my own, devised many years ago, which I have always found excellent; I call it the S.A.A. mixture (sublimate alcohol-acetic). It differs from Schaudinn's fluid in that, instead of adding only a few drops of acetic, I add five per cent as I long ago came to the conclusion that Schaudinn's fluid does not contain enough acetic. The films were taken through the alcohols and stained by Heidenhain's iron-hæmatoxylin in the usual manner; eosin was generally used as a counter-stain. (To avoid repetition I refer subsequently to preparations made according to method (*a*) as smears; and to those made according to method (*b*) as films, except where stated otherwise.)

Now and again, however, for purposes of comparison I have stained a dried smear, after fixation with alcohol, by iron-hæmatoxylin; and on the other hand, a wet-fixed film by Giemsa.

In making smears and films from the spleen and bone-marrow, it is important not to smear the substance of the organ directly along the slide or cover-slip, but to tease up small portions thoroughly with a pair of very fine needles, in salt-citrate solution, and then make the smear or film in the usual manner. By doing this the cells are not only nicely separated, but the great majority of them are of normal appearance and intact, not ruptured or squashed-out (in the case of smears, the large megakaryocytes are at times unavoidably flattened to some extent). But in a smear made directly from an organ, frequently nothing but a "mush" results, the nuclei alone standing out from a common matrix of ground-substance representing the cytoplasm of the squashed cells.

As a specific stain for chromatin I have followed the classic method of adding dilute, acidulated methyl-green to the fresh unfixed cells or elements.

BLOOD-PLATELETS : THEIR NATURE.

Some months ago my attention was drawn by my friend, Dr. Ledingham, to a recent paper by Herwerden[6], in which the author expressed the opinion that platelets possessed the characters of differentiated cells, i.e., containing nucleus and cytoplasm. I had not previously given these elements special consideration, but as soon as I gave any thought to the question, this view struck me from what I had already observed, as being most unlikely, so that I decided to study them myself more closely, little knowing the important results to which my observations would lead me.

The usual appearance of platelets in ordinary smears is so well known that only few words are here required. When seen in the condition most nearly resembling that in which they occur normally in the blood, the platelets are generally small, spherical or ovoid bodies,¹ about one-fifth to one-fourth the diameter of a red corpuscle. But they vary in size not only in different animals but also to some extent in the blood of one and the same individual. When stained a platelet appears (fig. 1) as a clump of granules of varying size, staining usually red (the shade of colour varies according to the Romanowsky modification used), and embedded in a faintly blue-staining ground-substance; this ground-substance is sometimes hardly distinguishable apart from the granular mass. Near the edge of a smear the platelets are often collected into a clump, in which the ground-substance has coalesced so as to appear as a common matrix containing the aggregations of platelet-granules.

The first thing to ascertain was whether the red-staining granules represent chromatin or not. On applying the methyl-green test the platelets remain entirely unstained, whereas the nuclei (only) of all the leucocytes are stained a bright green. This is conclusive of the absence of chromatin in the platelets; in spite of the red colour of their conspicuous granules.

I then proceeded to examine films, mounted after varying degrees of differentiation.

In films which have been only momentarily in the iron-alum, just to cleanse the film, practically no differentiation is effected (fig. 2*a*). The red corpuscles are a dull black, more or less uniformly except in the thinner part of each, where the colour is greyish-black. The platelets are also uniformly black, or greyish-black in the case of the smallest ones; in the case of the largest, and particularly where a few are closely clumped together, the colour is an intense, bright black, almost a blue-black.

In a film which has been differentiated to the right extent as regards the leucocytes, and counterstained with eosin (fig. 2*b*), the nuclei of the leucocytes (of

¹ However rapidly the preparation, fresh or permanent, is examined or made, a few irregular or streaky forms can nearly always be found.

any one type) all show the same organized structure, though certain will be a shade darker, and others a trifle lighter, according as the hæmatoxylin has been rather less or rather more extracted.¹ On the other hand in the case both of the corpuscles and of the platelets, the uniformly diffused stain comes out entirely, but in a patchy manner. That is to say, in some cases, part of the corpuscle or platelet will still retain some of the stain, while the rest is free from it. In the case of the homogeneous substance of the corpuscle, the limit of the still-staining portion is ill-defined and shades gradually away. In the case of the finely granular substance of the platelet, the stained portion is rather better defined, but still patchy. And, of course, as more and more of the black stain is lost, the contrasting pink of the counterstain is more apparent.

Hence, while some large platelets, or compact clumps, are still black, others show little patches of varying size still black, the rest being pink; while yet others are entirely pink.

Thus in the platelet, as in the corpuscle, there is *no* definitely organized internal body, retaining its shape and structure, only becoming gradually fainter as the stain is extracted.

Impressed by the similarity between the corpuscles and the platelets, as regards the manner in which the black stain can be readily removed *in toto*, I de hæmoglobinized some smears, by laking the blood with water in the usual way, and then fixed them with absolute alcohol and stained with iron-hæmatoxylin. These smears, too, were merely rinsed for a second or two, with the alum, to clear away the excess of stain, so that they had no differentiation (fig. 3). No traces of the corpuscles are present, with the occasional exception of a faint ring, denoting the "skin." The nuclei of the leucocytes are wholly dense black, showing no structural differentiation. Their cytoplasm is of the customary pale, neutral tint. The platelets appear as uniformly pale bodies, their substance being very finely granular. There is no trace of the intense black stain about them. The substance which stains thus in ordinary films has been dissolved away in the laking, just as is the case with the hæmoglobin of the red corpuscles. Only, while the entire substance of the corpuscle has vanished, the substance of the platelet is left.

What could this black-staining substance be, which is present to a considerable extent in the platelet? It appeared to me most likely that it was some iron-compound, loosely associated with the substance of the platelet, but not intimately united with it, as is the iron in chromatin; a compound which was readily soluble in water, as is hæmoglobin. The question arose whether hæmatoxylin-stains (e.g., iron-hæmatoxylin, or Delafield's) could be regarded as indicating "masked" iron-compounds, or certain of them, by staining the elements containing such; in other words, whether it is on account of the presence of combined iron, in certain forms, that such elements stain by this method. MacCallum [7], dealing with iron-compounds in cells, says that "the hæma-

¹ It must be noted that, in any film, the degree of differentiation is rarely uniform throughout. Parts where the film is thicker tend to be less differentiated than others where it is thinner. Hence different individual cells or elements of the same type show slightly varying degrees in the amount of extraction of the stain.

toxylin stain" (this worker used Ehrlich's modification) "in the chromatin is always found to correspond in intensity, in the object stained and in the general distribution of the stain, with the blue reaction obtained in the other sections" (i.e., the Prussian-blue reaction used as the microchemical test). Similarly, the hæmoglobin of the red corpuscles is stained intensely, as already noted (and also, it may be added, when Delafield's modification is used). I was inclined, therefore, to answer this question provisionally in the affirmative, and also to regard the black-staining substance of the platelet as representing some iron-compound. I may mention here, that following Professor Boycott's kind advice, I have been able to demonstrate the presence of iron to a considerable extent in "platelet-cytoplasm," i.e., the cytoplasm from which the platelets are formed, by means of a definite microchemical test (*vide* below, p. 334).

I had therefore, a certain amount of light upon the nature of blood-platelets. They are not complete cells, because they possess nothing of the nature of a nucleus; they do not consist, at any rate, of unaltered nuclear material, because they have no chromatin. Platelets are bodies consisting of protoplasm—organized material, and not merely organic (e.g. some proteid), or it would be, in all probability, homogeneous—with which is associated a relatively considerable amount of intensely staining substance which I regarded provisionally as being some iron-compound.

BLOOD-PLATELETS: THEIR ORIGIN.

The latest work of which I knew at this stage, one dealing with both platelets and thrombocytes, was the valuable résumé of Werzberg [14]. This author sums up the question of origin in these words: "As regards true platelets it is now well-established (apart from the work of Wright) that they are derived from the contents of the reds." By the "reds" he means, it is to be gathered, the immature reds still containing the nucleus; and he does not definitely commit himself further. The only paper advocating the origin of the platelets from the megakaryocytes, of which I was then aware, was one of Wright's first papers [18], namely, the reference given by Werzberg. And the chief reason why I paid very little attention to the view of this author at the time was because of my opinion that the platelets contained a considerable amount of iron. MacCallum says (*loc. cit.*): "the presence of assimilated iron, apart from its occurrence in hæmoglobin and hæmatin, is an exceptional feature in the cytoplasm of the cells of the higher forms of animal life." The only exceptions, apart from hæmatoblasts, are yolk-containing cells, etc., and ferment-forming gland-cells. Hence I did not see at this stage, how platelets could possibly be derived from any kind of white blood-cell.

The information I had gained suggested, therefore, some connexion with the red blood-cell elements as being most probable. I was very doubtful about an origin directly from mature corpuscles, because it seemed to me that one can hardly regard the substance of the latter as being (any longer)

organized cytoplasm.¹ Otherwise when the hæmoglobin is dissolved away there ought to be some protoplasmic ground-substance left behind; whereas there are, at most, traces of a delicate enclosing "skin" or membrane.

For a time I was inclined to think that the platelets might be derived directly from the nuclei of the immature reds by their gradual breaking-up; whether while yet inside the cell or after extrusion therefrom, had to be ascertained. MacCallum (loc. cit.) has shown that the hæmoglobin is developed from the chromatin of the nucleus of the corpuscle-forming cells. And I thought it was quite possible that after the formation of the hæmoglobin was completed, the nucleus might continue to undergo alteration, with resulting disappearance of the remaining chromatin, separation of the iron-substance and eventual fragmentation into platelets. Holding this view tentatively, I proceeded to the study of the hæmatopoietic organs.

I will not weary readers by describing the history of this period. Suffice it to say that I went from bone-marrow to spleen and from spleen back again to bone-marrow in a search for indications. Both in the bone-marrow and spleen I found an abundance of immature reds (normoblasts), numerous free, extruded nuclei of these (fig. 5*b*), and here and there a nucleus in the act of being extruded; this act, however, is most difficult to catch. I saw no signs of the gradual break-up or dissolution of the nucleus in the developing corpuscle, and it was clear that the vast majority² at all events, of the nuclei are extruded intact and still possess chromatin. Neither could I see any indications of the alteration or fragmentation of these free nuclei; and all of them are very much larger than any individual platelet.

For a time, therefore, I was at a loss and incidentally turned to the study of the Kurloff-bodies, whose appearance in the smears I had been examining had intrigued me greatly. As soon as I saw these bodies in *films* I not only realized their nature and origin (see below), but was put on the right track in regard to the origin of the platelets. I at once concentrated my attention upon the macrophages (the large mononuclears, or "transitionals," and the megakaryocytes) and found that *the platelets represent certain products of the digestion of blood-cells and elements in*

¹ As they are certainly not complete cells, I restrict myself to the use of the words red corpuscle or corpusele, to distinguish them from complete blood-cells, such as the leucocytes.

² In smears of the blood of a purpuric guinea-pig, kindly given me by Dr. Bedson, here and there corpuseles (with hæmoglobin), can be found containing still small nuclear fragments (so-called Jolly bodies); and immature corpuseles possessing a nucleus which is apparently undergoing fragmentation in situ. It is possible that these small bodies may ultimately lose their chromatin and become platelets; but that this method of formation, if it occurs normally, only does so to a negligible extent, I am convinced by my study of the organs of normal animals.

the cytoplasm of the macrophages, together with a small portion of the disintegrating cytoplasm itself, abstracted from the cell.

THE SMALL MACROPHAGES (LARGE MONONUCLEARS AND
"TRANSITIONALS").

As there are rarely more than two or three blood-cell elements undergoing digestion at once in a large mononuclear, and the digestive vacuoles are nearly always separate from each other, the progress of the alteration and the concurrent appearance of the platelet-granules can be seen here much more distinctly than in the case of the megakaryocytes, in which the bulk of the cytoplasm is often in the condition of "platelet-cytoplasm." And although the large mononuclears perform their work individually in a much more modest and less demonstrative fashion, there can be no doubt, I think, that in the aggregate they must produce a considerable quantity of platelets because of their numbers.

The process usually known as "phagocytosis" goes on normally to an enormous extent in the spleen and bone-marrow, particularly of course in the former organ. In the circulating blood, macrophages containing ingested red corpuscles are rarely, if ever, met with normally. But in the case of some of my smears and films, especially of spleen-pulp, one has only to allow one's eye to traverse a few fields to see dozens of these cells, containing usually one to three, or even occasionally four or five corpuscles (cf. figs. 4a and 4b). Doubtless, the extent to which the process is going on varies slightly from time to time, according to the number of corpuscles becoming at the moment effete; for it may be inferred that normally¹ only the worn-out corpuscles will be eaten. In addition to the corpuscles, however, these cells take up the free nuclei of the immature reds; at times a cell can be seen containing both these kinds of element (fig. 4c). Old lymphocytes and polymorphs are very rarely eaten by the large mononuclears, but I have occasionally seen what I took to be a lymphocyte-nucleus in course of digestion. The red corpuscles must be taken up with extreme rapidity because I have never caught a large mononuclear which seemed to be in the act of engulfing one.

As regards the nature or meaning of the changes which occur during digestion, I am, unfortunately, unable to determine them. Such work comes within the province of biochemistry. All that I can do is to give some idea of the microscopical changes to be observed. It is highly probable, of course, that

¹ In this connexion the record of a fatal case of anæmia, by M. Rowley [11] is of great interest. Here phagocytosis of the corpuscles, "healthy" ones included, it would seem, was occurring abundantly in the general circulation. While the large mononuclears were chiefly engaged in this destruction, other leucocytes (polymorphs) also took part. It is to be noted that the authoress remarks incidentally, that the blood-platelets were enormously increased in number; and she observed them in the act of being cut off from the cytoplasm of the devouring cells.

some ferment or enzyme secreted by the nucleus is largely concerned in the process; we know this to be the case with many Protozoa, where the course of intracellular digestion has been studied. It is significant that an ingested corpuscle, in the digestive vacuole (often "virtual" rather than apparent) usually comes to lie very close to the nucleus, which, often, indeed, partially surrounds it.

The first change is that the hæmoglobin is rendered colourless—bleached, as it were—so that the corpuscle appears as a practically colourless vacuole. Next, in close association with the corpuscular substance, as a result of further change, the characteristic red granules are formed. At first these are very fine and minute, and stain only faintly red; often the appearance is more that of a very finely granular, pale red-staining substance. Subsequently the granules become discrete, much more prominent and stain deeper (cf. different stages in fig. 4, right-hand side). The vacuolar appearance is gradually lost as the assimilable products of the digestion are incorporated into the general cytoplasm. Unlike what is the case in the megakaryocytes, the red granules do not generally become uniformly dispersed throughout the cytoplasm, but tend to remain in more or less distinct clumps. The granules represent apparently the unassimilable residue of the digestion, and as such are more of the nature of metaplastic products—non-vital and passive—than an incorporated, essential constituent of the cytoplasm.

A most important point to note is that I have never seen the slightest indication of the formation of pigment-grains in connexion with this digestion of the corpuscular substance. Iron-containing pigment is, of course, not always formed in connexion with the true digestion of hæmoglobin by cells, e.g., parasites. Thus, while the malarial parasites produce pigment, *Piroplasma*, etc., the Hæmogregarines do not. Again, in cases where a parasite devours whole corpuscles, *Entamoeba histolytica* and *Balantidium* do not form pigment, whereas a blood-eating Ciliate from a whale, recently described by Woodcock and Lodge [17], under the name of *Hæmatophagus*, produces masses of pigment. Now, in both types of cases, i.e., the malarial parasites and *Hæmatophagus*, the production of the pigment-grains, *in situ*, at first fine and then becoming larger and more conspicuous, is always seen without the slightest difficulty. Nothing of the kind occurs during the digestion of the red corpuscles by large mononuclears.

Two conclusions follow, I consider, from this point. First, that the *iron of the hæmoglobin is retained in some form in the cytoplasm of the macrophage*; (and equally, of course, that derived from the chromatin of ingested nuclei). Secondly, that the grains and clumps of yellow (ferruginous) pigment, commonly occurring in the spleen, are *not* derived from the digestion of the red corpuscles, i.e., are not a direct product of "phagocytosis." How these pigment-masses result I cannot say; I can suggest that, in large part, they may be formed from the ultimate disintegration of the platelets themselves, and also perhaps, when red corpuscles or other elements disintegrate in the plasma.

It is noteworthy how rarely large mononuclears can be found containing any pigment, even though the smear contains large numbers of cells with corpuscles undergoing digestion. Contrast this scarcity with the abundance of pigment-containing macrophages in a malarial spleen. I consider that, far from getting rid of the iron (in the form of pigment), the mononuclears require this substance for the "platelet-cytoplasm," and it may be on this account, owing to the large

destruction of red corpuscles by the parasites, that these cells eat the pigment in cases of malaria.

To complete the consideration of the normal course of the digestion, after this digression, it may be added that, in the case of ingested nuclei, there is, of course, no hæmoglobin to consider. The nucleus seems at first to become denser and to stain, if anything, more deeply than it does normally. But as it undergoes digestion, and its structure becomes altered, it appears much looser in character and stains more lightly. Ultimately it becomes entirely granular, the granules staining bright red. It is, in fact, at times difficult to be sure whether a granular mass results from nuclear or corpuscular digestion; the granules in the former case tend to be rather more prominent.

The further behaviour of the macrophages and the actual production of platelets is better dealt with in relation to the megakaryocytes, where the process is on a much more comprehensive scale.

THE LARGE MACROPHAGES (MEGAKARYOCYTES).

The megakaryocytes are most remarkable cells. Though they are well-known I make no apology for considering them here in some detail.

According to Guieysse-Pelletier [5], a megakaryocyte begins life as an endothelial cell; this author has noted their origin in this manner in the case of white mice. A megakaryocyte, when quite young, is indistinguishable from a cell of large mononuclear or transitional type; and such cells, too, are regarded as being of endothelial origin. In my films a perfect series of transitions can be found between cells of large mononuclear character, with a lobed or indented nucleus, and huge, full-grown megakaryocytes (fig. 5). The megakaryocytes occur predominantly in the bone-marrow, but also in the spleen. But in my preparations of the latter organ, their occurrence is much more variable than in those of bone-marrow. This may be due to different preparations having been made, unthinkingly, from different parts of the spleen.

The outstanding features in the life-history of this form of cell is that cell-division, *as a whole*, is very largely inhibited, or in abeyance. The cells doubtless divide at times; but normally I should say only very exceptionally, unless when quite young. The end of a full-grown individual is not division and production of an entirely new cell-series, but death and disintegration.

The growth of a megakaryocyte is characterized by the continual increase in the amount of nuclear material. As the cell elaborates more and more nuclear substance (including chromatin), the nucleus becomes massive and lobed, and often undergoes division, unaccompanied by cytoplasmic partition. Thus a multinucleate condition results. In no other way can the many nuclei which several of these large cells possess, be adequately explained. It is quite likely, though this is only inference from the constrictions often to be observed in a nuclear mass, that the mode of division when unaccompanied by cytoplasmic division, is little more than that of simple fission. The separate nuclei in any one individual

are generally approximately equal in size and of the same slightly ovoid shape; they all have, moreover, an identical appearance, staining character, etc., which is different from that of the nuclei of the ingested cells.¹

The megakaryocytes eat chiefly leucocytes (polymorphs and lymphocytes), and the free nuclei of the normoblasts (figs. 5c and 5e); but in addition, red corpuscles are sometimes eaten (fig. 6). This hæmatophagic behaviour on the part of the megakaryocytes is always taking place, normally, in a quiet way. In any smear of bone-marrow or spleen, some of these huge cells can be found containing ingested cells, but the occurrence does not strike the eye in such a marked manner, of course, as does the "phagocytosis" by the large mononuclears. For one thing, there are not nearly so many megakaryocytes; and for another, the red cell-elements are mostly disposed of by the mononuclears, and, of course, these vastly predominate in number over the leucocytes. To show, however, the degree to which hæmatophagy may take place in the case of the megakaryocytes, on occasion, I have been most kindly allowed by Dr. Ledingham to have the photos taken which are reproduced in figs. 7 and 8, from the bone-marrow of a purpuric guinea-pig. (For description, see explanation of the figures.)

I have never seen any signs of pigment-grains in a megakaryocyte.

It is only from films that one can realize how extremely amœboid these great cells are. Many of them are caught showing blunt pseudopodial outgrowths of varying size and shape (fig. 5c). A megakaryocyte, in fact, resembles in several respects a large predatory Amœba! But herein lies an essential difference, namely, that the megakaryocyte is periodically engaged in *losing* its pseudopodia, whether large or small. How far this is an active (voluntary) process, or how far involuntary—a simple moving away of the cell, leaving eventually a portion of its cytoplasm broken off behind it—I am unable to say. I have an example of a megakaryocyte which shows a large pseudopodium with a pronounced constriction at its basal end, appearing as if it were in the act of being abstricted. These separated portions of megakaryocyte-cytoplasm, large and small, can be easily recognized in the smears.

Owing to this periodic loss of cytoplasm, there is at times considerable variation in the nucleo-cytoplasmic relation obtaining in these cells. While in some, there is an ample quantity of cytoplasm, in others, doubtless corresponding to a stage in the cell-life following upon the abstriction of large portions of platelet-cytoplasm, there is very little. At such a time, the cell consists mainly of this huge nuclear complex.

Further, at different periods, the quantity of chromatin contained in the

¹ It is a ridiculous hypothesis to suppose, as is the opinion of Guieysse-Pelletier (loc. cit.) that where there are multiple nuclei, the additional ones are actually the ingested nuclei. It is impossible to imagine that a foreign nucleus could be incorporated bodily and become a constituent, functioning organella of the strange cell. No doubt the extra nuclear material is built up from the assimilated substances derived from the nuclear food—but indirectly.

nuclei varies greatly; sometimes the nuclei are intensely stained; at other times (in the same film) they are so poor in chromatin that they are almost pallid (there is no question here of this great difference being due merely to varying differentiation). It is most instructive to see a cell with large, pale nuclei and little cytoplasm, in this extreme "hunger-phase," containing several recently ingested cells or nuclei, from the assimilated products of whose digestion it will build up its protoplasm anew (fig. 5e).

Practically the whole of the cytoplasm is potential "platelet-cytoplasm." The platelets result either from the break-up of the abstricted cytoplasmic fragments; or, especially in the case of older cells, approaching a worn-out condition, they may be separated off directly from the periphery of the cytoplasm, which has then a particularly frayed-out appearance (fig. 5d). The realization of the essential platelet-character of this cytoplasm is readily obtained from smears (or films stained by Giemsa).¹ The characteristic, small, red-staining granules are often distributed uniformly throughout the dense, blue-staining cytoplasm. Where the granules are very fine, the cytoplasm, for this reason, acquires almost a mauve tint. Near the periphery, and especially in a lobed portion, or pseudopodium, the granules become definitely aggregated into clumps (fig. 9). The larger, free masses also show this well-marked distinction. Even in small abstricted portions, containing only aggregations of granules, the cytoplasm is still more definite and more deeply blue-staining than is the case where clumps of platelets are seen in the blood. As the small masses break up into separate platelets, the cytoplasm probably breaks down still further, to become of the delicate and faint-staining character associated with the discrete platelets.

The end-stage in the life of a megakaryocyte occurs commonly in some of my films (fig. 5f). The large, extremely pallid nuclei are scattered about, two or three often being still connected together by a fine thread (nuclear membrane?); their cytoplasm has practically vanished, but there are usually a few stray tags still attached, some of which are almost resolved into platelets.

The behaviour of the cytoplasm of the large mononuclears is essentially comparable with that just described in the case of the megakaryocytes, only the process goes on in miniature. Small abstricted portions of the cytoplasm of these cells, containing aggregations of granules, occur in numbers (fig. 4d); but each such small mass will only break down usually into a few platelets.

I had worked out this whole question of the nature and origin of platelets—many of the drawings being actually made—before I ascertained that considerable confirmation of Wright's work had been already brought forward. Only a few

¹ As in so many other cases, the granules are unduly conspicuous after Giemsa; they are not at all prominent, apart from the general granular character of the cytoplasm, in films and have no particular affinity for hæmatoxylin (cf. fig. 5 with fig. 9).

days before beginning to write this article, I was steadily searching through the later volumes of the *Folia hæmatologica* with a view to finding what was the latest of Schilling-Torgau's ever-changing opinions upon the nature of the Kurloff-bodies, when I came across a paper by Hal Downey [4] upon the origin of platelets from megakaryocytes. Many of Downey's figures showing the abstriction of platelet-cytoplasm present just the same appearance that I have found in my preparations. And had I known at the commencement of my work of Downey's paper, I should certainly have studied the macrophages earlier than I did. As Downey somewhat pathetically remarks: "Wright's work has not been generally accepted, especially in Europe."

All credit, therefore, must be given to Wright and his followers for having determined the immediate origin of the platelets from megakaryocytes. Their view is correct, so far as it goes, but they have not taken into account what is, I consider, the most important factor in the process.

In the first place, Downey describes also the pinching-off of small buds of cytoplasm both from large mononuclears and from lymphocytes, but is of the opinion that neither of these types of cell gives rise to platelets. He is quite right as regards the lymphocytes; as will be seen in a moment, in connexion with the Kurloff-bodies, these leucocytes (which are, rather, microphages) *cannot* (normally) form platelets even if they become hæmatophagic. But the large mononuclears certainly can do so; it was from these particularly that I learnt the secret of the real origin. The mononuclears, and these only, are of essentially similar origin and character to the megakaryocytes. Both these macrophages not only feed in the same manner, *but are able to digest and assimilate the blood-elements taken up*; that is the important point.

And this is the point which Downey has entirely missed; he considers that the granules of the platelets are actually extruded from the nucleus, i.e., that they represent directly some nuclear material. I have not seen the slightest evidence of such extrusion in my preparations and this view is, I think, from what has been shown above, untenable. Some ferment or enzyme is doubtless secreted by the nucleus; but the granules are a by-product of the interaction between this ferment and the food-material undergoing digestion.

To sum up: *platelets are a direct result of hæmatophagy*; in my opinion, if the macrophages (large mononuclears and megakaryocytes) did not eat and digest blood-elements of all kinds there would be no platelets.

NOTE ON THE DETERMINATION OF THE PRESENCE OF "MASKED" IRON, BY MEANS OF THE PRUSSIAN-BLUE REACTION.

After considerable difficulty I have succeeded in obtaining definite indications of the occurrence of iron, on the application of this microchemical test. Adopting first MacCallum's method of prior treatment with four per cent sulphuric acid-alcohol, I met with no success, either as regards platelet-cytoplasm or (it may be here mentioned) the Kurloff-bodies. Only the chromatin of the nuclei reacted

to the application of potassium ferricyanide + dil. HCl. It was evident, therefore, that the form in which the iron is combined in the cases under consideration, is almost as difficult to break down as it is in haemoglobin, although it is a different compound. However I came across a paper by Brown [3] on the liberation of iron from haemoglobin and allied compounds by means of hydrogen peroxide and after many trials I succeeded in getting this oxidation-method to work. Films and smears were placed in a fifteen per cent solution of this reagent (Merck's "Perhydrol," diluted with an equal volume of distilled water—the ordinary weak solution gives no result) for two, or even three days. They were then well washed with water and placed for five or six minutes in the ferricyanide-HCl mixture. The preparations were mounted without being "stained" in the ordinary way, because it must be remembered that the colour obtained by the reaction is much more delicate, as a whole, than the strong effects produced by staining with, say, iron-haematoxylin and eosin.

The blue is relatively deepest and strongest, of course, in the cells which are largest and of greatest depth (in wet-fixed films). Thus in the megakaryocytes, the blue of the cytoplasm stands out conspicuously from the surrounding cells. The peripheral nuclear ring (or rings), which contains most of the chromatin, is also strong blue, but the central part of the nucleus is distinctly pale, i.e., there is little or no iron in the nuclear sap. The cytoplasm of the large mononuclears is also definitely blue, but lighter than in the much larger megakaryocytes. On the other hand, the cytoplasm of the lymphocytes and polymorphonuclears is quite pale and, indeed, in these "unstained" preparations can often scarcely be made out at all. The red corpuscles themselves are a light blue, rather a greenish-blue: I should say the iron in the haemoglobin is the most difficult to liberate completely of any of the iron compounds considered. Owing to the delicacy of the reaction, the small, individual platelets are difficult to detect in a film. In a dried smear, however, especially in the "tails," these can readily be found and are seen to have a distinct coloration, comparable to that of the chromatin of the nuclei. (For some reason, in a dried smear, the chromatin does not react so markedly as it does in a film.) But, on the other hand, the cytoplasm of the polymorphs and lymphocytes is entirely colourless.

THE KIELIFF-BODIES.

The next question is, apart from the macrophages are any other of the blood-cells ever normally haemotrophic? There is an instance (at present regarded as a very exceptional instance) of another type of blood-cell, namely a lymphocyte, which behaves in this manner normally—that is to say, customarily¹.

¹ I think that bodies comparable in origin and nature to Kieliff-bodies may yet be found to occur in other cases. A comprehensive paper by Wersberg [15] on the comparative haematology of cold-blooded vertebrates, contains certain figures of inclusions in lymphocytes which, to my mind, are most suggestive of Kieliff-bodies. Again, Ballou, in an important paper [12] which is a mine of information in regard to the explanation of the various puzzles and difficulties liable to be met with in blood-examinations, states that he has encountered similar bodies in the "mononuclears" of cats and fowls. One may certainly expect to find them occasionally in other rodents, but they will be in lymphocytes, not in true large mononuclears.

The enigmatical bodies known as the Kurloff-bodies, which occur very frequently in the lymphocytes (usually the large ones) of the guinea-pig, have been the subject of many papers. The two or three most recent, of which I am aware, are those by Pappenheim [10], Schilling-Torgau [12] and Schulhof [13], to which readers are referred for consideration of the earlier work. From these papers, it is clear that there are at present two opposed views as to the nature of the Kurloff-bodies. (1) They are either, as a whole, parasites, or else they indicate very minute parasites enveloped by a secretion-product of the cell, or by extruded nucleolar material, the actual parasites not being discernable; in the latter case, they are regarded as Chlamydozoa of some kind, surrounded by a "mantle." (2) They are cell-inclusions, consisting only of secretion-products of the cell itself—"autogenous physiological formations." Schilling-Torgau's latest opinion seems to be that they are of "archoplasmic" nature.¹ All the authors named hold the second view and have now, at all events, discarded the parasitic hypothesis.

Only brief reference is necessary to the usual well-known appearance of these bodies, as seen in an ordinary smear (fig. 10). Within a clear space or vacuole, in the cytoplasm of the cell, elements occur of very varied form and size, which stain homogeneously an intense red. There may be only a single fairly large round mass, or two or three smaller ones; or there may be numerous inclusions in the form of curving threads or rods, short or long; or, finally, there may be merely a number of granules of varying size. The size of the vacuole itself, i.e., of the whole Kurloff-body, also varies greatly and without reference to the condition of the contained elements.

These bodies are to be found both in the circulating blood and also in the bone-marrow; in some animals they are numerous, whereas in others they are scanty. But they always occur most abundantly, by far, in the spleen. And as soon as I examined *films* of this organ, I found that most of the Kurloff-bodies had *an appearance identical with that of the red corpuscles*.

In a preparation differentiated to the right extent for the leucocyte-nuclei, most of the corpuscles (fig. 11a) still contain a good deal of the iron-hæmatoxylin not yet extracted from the central part; a smaller or larger zone around the periphery (according as more or less hæmatoxylin is left in the middle) is stained red with the eosin. From other corpuscles the black stain has been nearly all extracted, only a few little patches, especially in crenated corpuscles, being left in. Scarcely any corpuscles happen to be entirely red. Most of the spherical Kurloff-bodies are stained as a whole in just the same manner (fig. 11). They

¹ Balfour (loc. cit.) remarks that "Schilling's work goes to show that they" (i.e., the Kurloff-bodies) "are merely phagocytéd structures." Balfour has grasped the truth there, but it is not quite clear to me whether this is his own opinion of Schilling's (Schilling-Torgau's) work, or whether he means that Schilling himself has come to this conclusion. I think the former must be the case, because I cannot find any reference by Schilling himself to such a view. And in the paper cited in the text, in which he tabulates his annual changes of view for several years past, there is no mention of such a possible explanation of these bodies.

show a central part, larger or smaller, stained black,¹ the periphery of this area being often fuzzy or indefinite; and an outer zone stained red. In all cases, equally as in the case of the corpuscles, some homogeneous substance is being stained. Varying degrees in the loss of the black stain are seen, until some of the larger Kurloff-bodies are left with none. In the case of small bodies, those about the size of a red corpuscle, the black stain has invariably gone. Sometimes there may be two Kurloff-bodies in one lymphocyte on opposite sides of the nucleus.

Here and there in the large bodies from which all the black stain has been extracted, pale unstained elements can be seen of varying size and shape.

What an entirely different picture is here presented from that found in ordinary smears. Its manifest explanation is this. The large clear space in which the intense red-staining inclusions occur, in smears, is in reality filled with a uniform substance which stains by iron-hæmatoxylin or eosin exactly as does the hæmoglobin of the corpuscles. The substance is most probably liquid in state, because of the spherical shape of the Kurloff-bodies in films. On the other hand the remarkable inclusions themselves have little or no affinity for either hæmatoxylin or eosin. (At times there is the faintest indication of greyish-black around their margin, cf. under the Negri-bodies, to be dealt with in the December number.) Kurloff-bodies occurring free (fig. 11) can indeed only be distinguished from corpuscles when they are of larger size than the latter (unless the pale inclusions happen to show through the eosin).

Why do not the homogeneous contents of the "vacuole" which provide so striking a feature in the films, appear stained in ordinary Giemsa-smears? I think the reason is because the contents are no longer there. In the first place many of the Kurloff-bodies are ruptured (or rather the delicate cytoplasmic envelope enclosing them is) as the smear dries; and frequently some of the inclusions are seen partly or altogether outside the "vacuole." And the contents have most probably been washed away during the process of staining, etc. In wet-fixation, on the other hand, there is no drying and no rupture, and the cytoplasmic envelope is preserved intact.

A film stained by Giemsa is most instructive, and provides convincing evidence of the relation between the Kurloff-bodies and red corpuscles, if the particular film contains very early stages in the formation of the bodies (a spleen-film is most likely for this purpose). In such a case the contents of certain of the "vacuoles"—varying in size according as they represent one, two or perhaps three, recently ingested corpuscles—are still distinctly yellow in tint, with possibly the merest trace of pink added—*unmistakably of corpuscular origin*. In others the contents are definitely a pale rose colour; this appearance indicates a stage in the progress of the alteration. Lastly, in the fully formed Kurloff-bodies the whole contents

¹ It must be emphasized that there is no question of this black-staining area representing any nuclear body, any more than in the case of the corpuscles; it is entirely a question of the degree of extraction of the stain from the thicker central part.

are stained uniformly a strong, bright, rose-colour; the contained inclusions, also here quite colourless (unstained), are usually though not always (cf. fig. 12a) hidden. Thus a perfectly corresponding picture is obtained to that seen in films stained in the usual manner.

I have obtained this rose-staining in an ordinary *dried* smear in the following manner: the smear is made and left aside for some days unfixed and untreated in any way, and then fixed with absolute alcohol and stained by Giemsa. In one case I divided a smear, freshly made, into two halves by a grease-pencil mark; I fixed and stained one half at once in the usual manner, and the other not until two or three weeks later. The Kurloff-bodies in the two halves appear entirely different and would not be taken for the same structure. In the one half nothing but the strongly stained inclusions of the customary varied form; in the other the whole body a mass of rose-colour. Long exposure to the air must in some way alter the composition of the originally liquid contents of the "vacuole," "fixing" this substance as it were, and preventing it from being dissolved or washed away by the alcohol or water subsequently.

An interesting point (in relation to the Negri-bodies) is that, in smears thus left awhile the Kurloff-bodies may show, in addition to the rose-coloured mass, numerous granulations (fig. 12b); these are dark red in colour and tend to be more superficial in disposition, i.e., near the periphery. They do not represent, it must be noted, the granular inclusions in the "vacuole" of some Kurloff-bodies as seen in an ordinary smear. These granules are not noticeable in *films* however stained.

I have been fortunate enough to find what I regard as definite indications of the very recent ingestion of a corpuscle and of a portion of a corpuscle (at any rate smaller than the average size)¹ by a short pseudopodium of a lymphocyte. In one instance there is already a large Kurloff-body in the lymphocyte, and this additional small quantity of corpuscular material would undoubtedly have been added to it (fig. 11). In this manner the Kurloff-bodies may increase in size. The cell continues to take up corpuscles and as a rule, instead of remaining separate, *as is nearly always the case with the corresponding ingestion in large mononuclears*, all go to form one large body. In one instance a lymphocyte is seen which has ingested two corpuscles separately and the two masses are on the point of uniting into one.

In the other case a lymphocyte has just ingested a corpuscle by means of a short pseudopodium. The altering corpuscle is still some little distance from the nucleus, which almost invariably becomes ultimately partially wrapped round the Kurloff-body.

Only red corpuscles are eaten. I have never seen the slightest indication of an ingested nucleus with its recognizable structure, and of course its chromatin staining in the usual manner.

It may be asked, how do I know that I am dealing invariably with lymphocytes and not with large mononuclears which have just ingested a corpuscle in the usual course? Firstly, by the characters of the nucleus and cytoplasm, the

¹ Small corpuscles, or what are portions of broken-down corpuscles, occur commonly in the spleen preparations.

distinctions between which in the two cases, are shown particularly well in my films made in the customary way. Especially the greater quantity and the granular pink-staining character of the cytoplasm of the large mononuclears contrasts definitely with the small quantity (usually) and the pale staining, fine character of the cytoplasm of the lymphocytes. Further, the point just alluded to, *re* the different disposition of the included corpuscles in the two cases. Lastly, an interesting point is that recently ingested corpuscles in large mononuclears which still retain their hæmoglobin, always stain uniformly black after iron-hæmatoxylin (fig. 9*b*) ; that is, none of this stain has been extracted—unlike what is the case with the free corpuscles. On the other hand Kurloff-bodies (or corpuscles becoming altered into such), of the size of a single corpuscle are invariably red entirely. It is only the larger bodies, with a correspondingly greater amount of the substance filling the “vacuole,” in which more or less of the black may be still left. For some reason this substance in a Kurloff-body does not retain the black stain quite so firmly as does the corresponding quantity of hæmoglobin.

What then are the characteristic “inclusions” which appear pale in films and yet stain so strongly with Giemsa? It was already clear that they did not consist of chromatin, but I applied the methyl-green test to see their behaviour under the action of this stain. I found that they stained yellowish, a quite different colour from the bright green of the nuclei ; and they stood out well from the colourless “vacuole” in which they lay. These inclusions are definite homogeneous elements, not of chromatinic nature, which appear in smears and films in just the same various forms in which they occur naturally. In one case after I had treated one half of a freshly made smear in the above manner, I fixed this same half in alcohol and stained it with Giemsa, and in particular Kurloff-bodies whose position I had noted the appearance of the inclusions was precisely the same.

The whole explanation of the Kurloff-bodies is undoubtedly as follows : *they are the result of an unsuccessful attempt on the part of the lymphocytes of the guinea-pig to digest red corpuscles upon which these cells have a predilection for feeding.*¹ The nature of the alteration which takes place is quite different from that which occurs when the hæmoglobin is properly digested by the macrophages ; with the exception that neither in this case is any pigment produced. By the action of some ferment the hæmoglobin is broken down to a certain extent. This is, however, the only share the lymphocyte takes in the production of the Kurloff-body ; and this is, apparently, as far as it can go in the direction of metabolizing this food. The alteration in composition begins very rapidly, much more so than in the case of the digestion of corpuscles by large mononuclears. Hence the earliest stages are very difficult to find.

¹ Probably in this case also only, or mainly, effete corpuscles are taken up. The abundant occurrence of the Kurloff-bodies in the spleen suggests that this is so.

As with the black-staining substance in the platelets, so in the case of the homogeneous contents of the "vacuole," which stain uniformly black with iron-hæmatoxylin (or purple with Delafield), and which are also intensely eosinophil, I inferred that this substance contains the iron of the hæmoglobin, and not the pale inclusions. Here, too, this view is shown to be correct on the application of the microchemical test, as above described (p. 334). In films, the contents of the "vacuole" stand out a conspicuous, uniform blue, even more marked than is the chromatin of the nucleus, and completely hiding the colourless inclusions. The colour is deepest in the larger Kurloff-bodies, owing, of course, to their greater size. Judging from the degree of the reaction, I should say that the iron-compound here is one from which the "masked" iron is liberated more readily than in the case of the iron-compound present in platelet-cytoplasm.

The principal difference, therefore, between the digestion of the hæmoglobin by the macrophages, and its alteration into a Kurloff-body by the lymphocytes is as follows: In the former case, the hæmoglobin is metabolized entirely, the iron becoming incorporated into or, at any rate, associated with the cytoplasm in some still complex proteid combination. In the Kurloff-body, the iron is at once split off from a considerable part, at all events, of the protein-substance which helped to constitute the hæmoglobin; though even in the liquid contents of the "vacuole," the iron is still more "masked" than "free." The characteristic, red-staining inclusions (after Giemsa) represent this remaining proteid, which may be itself the product of some interaction between the original proteid and the "digestive" ferment.

This difference in the result of the hæmatophagic mode of behaviour explains, I think, why the formation of the Kurloff-body is so much more rapid a process than the digestion of the red corpuscles by the large mononuclears, which according to Boycott and others, probably takes anything up to a week to complete. In this latter case, the whole of the hæmoglobin, iron included, has to be metabolized in such a way that it can be assimilated. The lymphocyte, on the other hand, is apparently quite unable to assimilate and make use of this food. There the resultant Kurloff-body remains, as a cytoplasmic inclusion, increasing in size as more corpuscles are ingested and added to the mass: it never becomes less, by gradual absorption into the cytoplasm. The lymphocyte, it must be inferred, continues to take in nutriment by osmosis from the plasma. On the other hand, the Kurloff-body does not seem to harm the cell in any way, which can, apparently, divide while containing one (fig. 12*b*). When the mass has become of an inconvenient size, it is simply abstricted; this may indeed happen before the Kurloff-body has become anything like the full size to which it may attain; and it probably always happens if the lymphocyte divides.

The Kurloff-bodies occur free, that is, separated from the lymphocytes in which they have been formed. I have seen such free bodies occasionally in the peripheral blood; and in spleen-preparations, both smears and

films (fig. 11), they occur commonly and of varying size. These free bodies show a definite limit to the "vacuole"; in smears this border is often stained faintly blue. Doubtless, a minute portion of the cytoplasm of the lymphocyte, in the form of a delicate enclosing envelope, is abstricted along with the Kurloff-body. Ultimately these free bodies and their contents most probably break up altogether and are dissipated in the blood.

I have not been able to determine whether there is any definite sequence of change in the form and number of the masses and granules comprising the inclusions; or whether the varied appearances result, in the main, from the manner in which the proteid is separated (or formed) from the hæmoglobin. From a comparison of corresponding smears and films, where the latter show very early stages (corpuscular masses still yellow in tint), I rather think that, in the early stage, there is a finely granular condition of the inclusions. If I am correct, the larger masses and curved rods, etc., are perhaps formed by coalescence in various ways of the original granules.

II.—HÆMATOPHAGY AS A PATHOLOGICAL OCCURRENCE.

We have seen that the lymphocytes of the guinea-pig customarily devour red corpuscles, but, unlike the macrophages, are unable to assimilate this food, only altering it into a Kurloff-body. Nevertheless, the fact that these cells may be hæmatophagic immediately suggested to my mind the possibility of certain other types of cells—other cells than blood-cells—behaving similarly, *under abnormal conditions*. The Kurloff-bodies have often been compared, in a general way, with Guarnieri's bodies, Negri's bodies and similar formations; I mean, as being not necessarily identical, but of the same order of enigmatical structure. I realized that there was nothing inherently improbable in the idea of a tissue-cell, under the influence of some powerful, exciting stimulus, such as a virulent toxin, reverting to the primitive mode of behaviour found, for instance, in a large number of unicellular animals (Protozoa), that, namely, of ingesting "solid" food.

THE NEGRI-BODIES.

The Negri-bodies have been so well described that not much need be said about them by way of introduction. They are characteristic inclusions occurring in cells of the nervous system in hydrophobia. They occur commonly in the Purkinje cells of the cerebellum, and especially in the cells of the *cornu Ammonis* (*Hippocampus major*). Readers may be particularly referred to Negri's own works [8 and 9]. As to their nature, the "Chlamydozoan" view is very widely held. This is, that the conspicuous bodies themselves represent extruded nucleolar matter, enveloping the actual minute, parasitic elements. The usual complicated type of life-cycle has been described.

Acton and Harvey [1], alone among recent workers, so far as I am aware, take the view that the Negri-bodies are the result of an extrusion of nucleolar matter, in response to the exciting stimulus, but not in connexion with any parasite; that is, that no parasite is concerned, the "extrusion being the result

of katabolic changes in the nerve-cell caused by the action of the rabies-virus.' But the chief merit of the work of these authors is that they have been able to find bodies similar to Negri-bodies, in the nerve-cells of animals subjected to various other stimuli, such as viper-venom, emulsion of *Bacillus pyocyaneus*, and so on.

Among my preparations of the Negri-bodies are certain which were kindly given by Negri himself, some years ago, through the instrumentality of Dr. Visentini. One of these is an ordinary Giemsa-smear, and I will describe this first, as the appearances to be observed are exceedingly instructive. It must be borne in mind that the smear is more or less a "mush" of cell-substance. There is a common matrix, which consists of the cytoplasm of the cells which has run together, as it were, so that their individual outlines are usually indistinguishable. The smear itself, however, is very well fixed and stained. But it follows that, from the smear alone, it cannot be said definitely what was intracellular, and what was extracellular; where I indicate which, in my opinion, of the two conditions was the case with respect to any particular body, I am having regard also to the situation of the corresponding forms, as seen in sections.

Immersed in this ground-substance are various organellæ and bodies of perfectly definite nature. Narrow blood-capillaries (fig. 13a), of varying length, traverse it in different directions; these end abruptly, with open ends, and have probably been broken in making the smear. These capillaries contain more or fewer red corpuscles, which appear yellow (unstained). The corpuscles are often flattened laterally and slightly elongated, owing to the narrowness of the capillary; moreover, the adjacent ends of two corpuscles, in contact, are often indistinguishable. Corpuscles, identical in appearance, also occur free throughout the matrix itself. Scattered about are the neuroglial nuclei and also Negri-bodies, the latter being more common in some places than in others. They are of varying form, size and appearance.

A regular series of transitions can be found between what are undoubtedly red corpuscles, or corpuscular masses, and fully formed Negri-bodies. In the first place, quite short segments of a capillary are occasionally seen, narrow as usual, but completely rounded off at each end, by the delicate enclosing wall, which stains always red. In these isolated segments, the enclosed corpuscles are stained a greenish-blue, with perhaps a tint of yellow; that is to say, some alteration is already occurring in such cases which tends to produce a kind of polychromatophilic staining. In addition, ovoid masses occur (fig. 13), scattered about, varying in size from that of a single corpuscle to that corresponding to three or four, indistinguishably united. These masses all stain in a greenish-blue manner and represent equally blood corpuscles or corpuscular masses. In the same field may be seen such bodies and also normal, unaltered yellow corpuscles. The larger masses are invariably surrounded by a delicate red-staining sheath; there is no question of this being merely a deposit of stain, because it is

never present around free, unaltered corpuscles. Occasionally, these bodies are narrow and elongated. In some of the masses, fine granules, faintly red, are present, even while there is still a tint of green in the general staining; in others, the granules are much more prominent and stain more deeply and the substance of the altered corpuscles is definitely light blue. In one case, of a fairly large ovoid mass, with well-marked granulations, most of the ground-substance is blue, but near one end there is a pure yellow area, i.e., a still unaltered corpuscular fragment, incorporated in the mass (fig. 13*b*). Lastly, in others again, discrete, usually round, light-red staining areas are developed in the blue substance; there may be only one or two, or in larger bodies several of these.

Thus, finally, the stage of the fully-formed Negri-body is reached, as depicted by Negri from Giemsa-smears (fig. 13*d*); the larger, homogeneous, light red elements correspond to the "grosse Innenformationen," the small, dark red, mostly more superficial elements are the "kleine Innenformationen."

Bearing in mind the above reservation, I consider, nevertheless, that not only the polychromatophilic alteration of the corpuscles, but the whole transformation into a Negri-body, may, on occasion, take place really extracellularly, while the blood is still in a narrow, capillary segment. In one instance in a segment about ten times as long as broad, i.e., much longer and narrower than any ovoid mass I have found in a cell (in sections), rather less than half is occupied by an oblong, faintly yellow mass, representing probably two corpuscles: the other, larger half consists of a long, cigar-shaped Negri-body, with five of the larger inclusions (fig. 13*c*). As noted, many of the developing Negri-bodies have this elongated shape; and I think this is to be accounted for by their origin from short, broken capillary segments, the blood in which has been altered *en masse*. How these short portions of capillaries have been broken or separated I cannot say; but I consider that the larger, ovoid masses (definitely intracellular as seen from sections), result from the comprehensive ingestion of such short segments, the pink-staining sheath being probably the delicate wall, and the whole being moulded by the cell into a more or less ovoid shape. Where alteration, to a greater or less extent, takes place extracellularly, it must be inferred that the ferment can be poured out of the cell.

In sections stained by Mann's method, the preparation also being one which was kindly given by Negri, the red corpuscles, here again, are often compressed and tend to be crinkled lengthwise; thus they have a remarkably refringent appearance (fig. 14*A, c*). They stain a bright yellow-pink. It is important to note that isolated corpuscles—quite separate from a capillary—can be found without difficulty. These are undoubtedly *in situ*, and their occurrence free points to disorganization of some of the capillaries. Small Negri-bodies are just about the same size as a corpuscle and are also often *similarly elongated*. These small bodies now and again show one or two colourless, rather refringent elements inside them, these inclusions being the so-called "vacuoles." In the larger ovoid bodies, these inclusions are more numerous and some of them larger. The colour of the whole Negri-body is a bright red, lighter in the smaller ones and darker (stronger) in the larger ones. An elongated corpuscle becoming

altered into a Negri-body can be distinguished from an unaffected corpuscle only by its slightly different staining appearance, unless a small clear area has already been formed. On the other hand, the nucleoli of the nerve-cell nuclei are all round, uniform in size and of the same shade of purple, this colour being quite different from that of the Negri-bodies. (A very similar picture is shown in Muir and Ritchie's "Manual of Bacteriology," 1913, pl. 4, fig. 16, from material given by Harvey and stained with methylene-blue and eosin.)

Lastly, I compared some sections made by myself which were stained by iron-hæmatoxylin and eosin. In these, the iron-hæmatoxylin has been almost completely extracted from the Negri-bodies which are uniformly stained with the eosin, excepting where the pale inclusions stand out (fig. 14B). But around these inclusions there is often the last trace of hæmatoxylin in the form of a fine greyish-black rim. That is to say, by this method of staining *the appearance of the Negri-body and of the Kurloff-body is essentially similar*. The nucleoli, on the other hand, are always an intense black; i.e., they *retain* the iron-hæmatoxylin much more firmly and suggest an organella of entirely different character, namely, a karyosome, or chromatin-containing nucleolus. (The nuclei themselves of the nerve-cells seem to be very poor in chromatin.)

There is not, in fact, the slightest evidence in my sections suggesting any connexion between the nucleolus and the Negri-body. None of the nucleoli show the least signs of activity. They are uniform in size and appearance, both in cells containing a Negri-body and in cells which do not; and, as remarked, *whichever way they are stained* they are quite different from the Negri-bodies. Even if I had not been able to demonstrate actual stages in the alteration of corpuscles into a Negri-body, the nucleolar-extrusion hypothesis would have, I consider, nothing in its favour, whether regarded as a cloak to hide a "Chlamydozoan" or not.

It will be apparent that these varied appearances can all be readily correlated with those shown by the Kurloff-bodies when correspondingly stained. It must be remembered, however, that the hæmatophages concerned in the two cases are, respectively, cells of quite different order; in the one case a nerve-cell, in the other a lymphocyte. Consequently, it may well be expected that both the exact nature of the ferment secreted and the alteration produced in the ingested corpuscles as a result of the attempted digestion will be rather different in the two cases.

To take first the main points of agreement in both cases, only red corpuscles are eaten. *The fundamental structure of both bodies is the same* and consists of two chief components: (1) A homogeneous substance, filling the body, in which are included (2) elements, or "large inner formations," which are of varying size but usually round, in the case of Negri-bodies, and not only of varying size but of most varied form, in the case of Kurloff-bodies. In smears stained by Giemsa, these inclusions are a light, bright red in the Negri-bodies, and a rather darker red in the Kurloff-bodies. In films or sections, on the other hand, the inclusions remain colourless (unstained) in both cases.

The chief difference is due to the iron-compound, separated from the protein elements, being in a somewhat different condition in the two cases.

In the Kurloff-body, it is in a more liquid condition, and constitutes a practically spherical globule. In the Negri-body, the corresponding substance is more solid and may retain the shape of the original corpuscle, or corpuscular mass from which it is formed, until it is moulded by the hæmatophage into an ovoid shape. Further, whereas in the Kurloff-body, this substance stains a rose-colour by Giemsa, it stains a light blue in the Negri-body; i.e., it corresponds more to the polychromatophilic type of staining of hæmoglobin, in an altered condition.

In addition to the two principal constituents, granulations occur in the Negri-body (the "small inner formations"), but they are only conspicuous in Giemsa smears, doubtless rendered unduly prominent by this stain. When considering the Kurloff-bodies, I mentioned that occasionally, in smears where the contents of the "vacuole" have been retained (see p. 16) I have observed corresponding granules, in addition to the uniform, rose-coloured mass.

The varying size of the Negri-body depends entirely on the amount of corpuscular material ingested by the nerve cell, just in the same way as does the size of the Kurloff-body. *There is no growth*, or increase in size *per se*. The smallest Negri-bodies result from the ingestion of a single corpuscle (or even a fragment of one—just as a large mononuclear, or a lymphocyte may take up a portion of one); the largest, from the comprehensive ingestion of a mass of three or four corpuscles; and in one and the same cell there may be examples of both (cf. fig. 14B with fig. 11).

Here, too, it would appear that the hæmatophage is unable to assimilate this altered but only partially "digested" food. In my preparations I have not found the later stages depicted by Negri. From these, it is most probable, however, that the substance of the Negri-body breaks up into small fragments, "gemmules," which are ultimately dissipated—it may be when the nerve-cell is killed, or they may be expelled.

THE "CHLAMYDOZOA."

It is seen, therefore, that under the influence of the violent stimulus occurring in hydrophobia, even such highly specialized cells as nerve-cells may become hæmatophagic. Now, Guarnieri's bodies, occurring in the epithelial cells in small-pox and vaccinia, are *generally agreed to be bodies of similar character and to originate in the same manner as the Negri-bodies*. They, equally, have been included in the "Chlamydozoa," under another imposing name. I have not myself actually studied Guarnieri's bodies, but there can be, I consider, no doubt whatever, that these, too, are simply the result of epithelial cells becoming hæmatophagic,¹ with consequences of the same order; i.e., alteration,

¹ It must be noted that I say "hæmatophagic," i.e., blood-eating; it may well be that the epithelial cells do not restrict themselves to red corpuscles, but eat leucocytes also. In that case the alteration of the nuclear material would give a somewhat different result again. It is significant that various workers have already thus explained Guarnieri's bodies, e.g., Ewing who regarded them as derived from red corpuscles, and Ferroni and Massari and also Salmon, who derived the inclusions from leucocytes.

but not digestion and assimilation of the ingested material. Some of the "phases" described, whether intracytoplasmic, as in vaccinia, or intranuclear, in small-pox, are almost identical in appearance with Negri's bodies and are certainly to be regarded as similar formations.

And the same explanation may also apply to the case of the bodies in "*molluscum contagiosum*," trachoma, Mallory's bodies in scarlet fever, etc. In short, I wish to express my considered opinion that Prowazek's entire conception of the Chlamydozoa, with their "elementary corpuscles," "initial bodies" and all the complicated phases of their life-cycle, will be proved to lack reality and must be banished as an illusion. At any rate, there are no such parasites or parasite-complexes as "*Lymphocytozoon cobayæ*," "*Leucocytozoon (sic !)* *syphilidis*" and many of the intracellular "phases" intercalated by certain authors into the life-cycle of *Treponema pallidum*, "*Neuroryctes hydrophobie*" and "*Cytoryctes variolæ* and *vacciniæ*."

THE "RICKETTSIA"-BODIES IN TYPHUS, ETC.

Lastly, I wish to suggest that the parasites which have been described under the name of *Rickettsia*, occurring in typhus, and the very similar ones known as *Dermacentroxenus*, in Rocky Mountain spotted fever, a closely allied disease, are also to be regarded as *granular formations produced as a result of hæmatophagy*; in other words, that the minute bodies hitherto found in cases of these diseases and placed in the above category have, in reality, nothing of a parasitic or organismal nature about them.

I have not worked at these bodies myself, but as a result of my study on hæmatophagy I am in a position to put forward certain reasons which seem to me to indicate a strong likelihood of the view now advanced; these reasons are suggested by consideration of some recent papers dealing with the subject of typhus, or typhus-like diseases, and the Rickettsias, and I think they are worth serious attention and investigation on the part of those interested in this question.

In the first place, it is important to remember that the essentially characteristic lesion in this type of disease is not one of the skin, or of nervous tissue, as in the case of the "Chlamydozoan" diseases above referred to; *it is one of the vascular tissue itself*. Thus Wolbach, in his account of Rocky Mountain spotted fever (*Journal Medical Research*, 41, 1919, p. 1) states as follows: "The lesions of the blood-vessels are due to the presence of the parasite and constitute the distinctive pathology of the disease and warrant the definition, 'an acute specific, infective endangiitis, chiefly of the peripheral blood-vessels.'" "The lesions are at first essentially proliferative (endothelium), followed by necrosis of small groups of cells, and the chief cellular reaction, both locally in response to the presence of the parasite, and in general, presumably in response to toxins, is endothelial." Again, Wolbach and Todd, dealing with *typhus exanthematicus* in Mexico (*Ann. Inst. Pasteur*, 34, 1920, p. 153), write as follows

“La lésion consiste essentiellement dans une réaction de prolifération de l'endothélium vasculaire, bientôt suivie d'une infiltration de leucocytes polynucléaires dans la paroi cellulaire . . . La présence des cellules mononucléaires que l'on observe, en migration dans les parois des vaisseaux sanguins, nous amène à cette conclusion que les cellules mononucléaires des infiltrations périvasculaires sont d'origine endothéliale.” Finally, Stevenson and Balfour, in their recent summary of the histo-pathology of typhus (*Journal Path. Bact.*, 24, 1921, July, p. 289), say that it is now recognized that typhus is really a systemic disease of the smaller arteries and capillaries and that the vessel-changes are not limited to the skin, but occur in all the viscera.

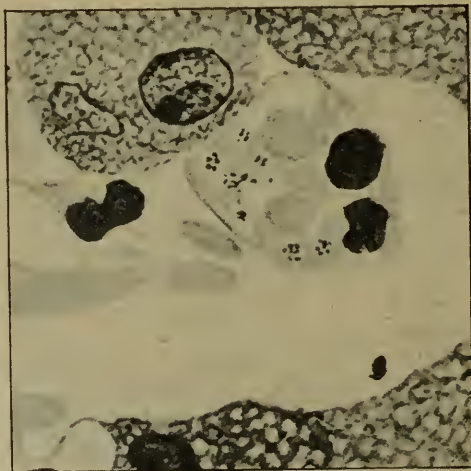


TEXT-FIG. 1.—Early vascular lesions in Mexican typhus, showing the parasites in capillary cells (above), and in the cells of a small vein (below). (From Wolbach and Todd.)

Further, just in the same way as the relatively large “parasitic formations” are associated with the chief foci of the disease in the case of “Chlamydozoan” diseases, e.g., Guarnieri’s bodies in the epithelium, Negri’s bodies in the nerve-cells, etc., so in typhus, the minute bodies which have been found hitherto and regarded as the causative organism occur mainly in cells of the vascular tissue—endothelial cells. Thus, Wolbach, in addition to the first sentence above quoted, speaks of the remarkable specificity of the parasite for the peripheral blood-vessels, and throughout his case-reports refers frequently to the finding of numerous parasites in endothelial cells and large mononuclears. Again, Wolbach and Todd say that, in Mexican typhus, “ils (les parasites) apparaissent en larges placards localisés exclusivement dans les cellules endothéliales” (cf.

text-fig. 1). And Kuczynski found large aggregations in the Kupffer (endothelial) cells of the liver, in cases of typhus.

Now, in my opinion, certain remarks of Stevenson and Balfour are most suggestive in this connexion. In the first place, these authors are undoubtedly hesitating whether to regard, as *Rickettsia*-parasites, certain red-staining granules, which they themselves persistently refer to as "platelet-like bodies" or as "similar to blood-platelets," occurring in the phagocytic cells of the spleen-pulp, in those of the liver (endothelial or Kupffer-cells) and elsewhere. It is true they seem rather more inclined to pin their faith to certain blue-staining granules (often diplococcal in form) occurring in the walls of the vessels in the brain and lung, but they conclude as follows: "Although we have never found in the Kupffer-cells of the liver, the large aggregations of *Rickettsia*-bodies described by Kuczynski, it is possible that the platelet-like bodies above-mentioned are really of this nature. Here, as also in the spleen, they stain with Giemsa in the same way as do the *Rickettsia* of Kuczynski, taking on a red colour. Hence they present a contrast to the bodies found in the brain and lung."



TEXT-FIG. 2. —Endothelial (Kupffer) cell in liver containing small masses of red-staining granules resembling blood-platelets. There is also phagocytosis of red cells. From a case of typhus. (From Stevenson and Balfour.)

And they add the following sentence, with which I entirely agree: "Too much stress, however, must not be laid on the mere colour-reaction, as there can be little doubt that, in section-staining, the results given by the Giemsa-stain are variable, even when to all appearance the same technique has been employed." In my opinion, so far as can be judged from the excellent figures, the granules do represent similar formations, whether labelled platelet-like bodies or queried as *Rickettsia*. On the view I wish to put forward slight differences in form and size have little significance.

The next important point to note is that "phagocytosis" of red blood-corpuscles by endothelial cells and large mononuclear cells, in or associated with the capillaries, has been described as occurring on a considerable scale

(cf. Wolbach, and Stevenson and Balfour, l.c.). Text-fig. 2, taken from Stevenson and Balfour's fig. 7, pl. 13, shows a Kupffer-cell of the liver containing both "small masses of red-staining granules resembling blood-platelets," which correspond most probably to *Rickettsias* in the same situation, and ingested red corpuscles.

What I wish to suggest, therefore, will be, I think, apparent. It is that the granules hitherto described as *Rickettsia* are essentially nothing more or less than platelet-granules, a by-product of the digestion of red corpuscles and, it may be, of leucocytes as well.

In typhus-diseases, the virus attacks and stimulates (at any rate, at first) the endothelial cells; stimulates them to hæmatophagy, just as the viruses of smallpox and hydrophobia stimulate the respective tissue-cells attacked to the same mode of behaviour. In the case of typhus, however, we should not expect conspicuous "bodies," like Guarnieri's bodies and Negri's bodies, to be formed, as a result of an *unsuccessful* attempt to digest this food. The normal macrophages (megakaryocytes and large mononuclears) belong to the endothelial cell-type, from which they are derived. Therefore, on *a priori* grounds, if an endothelial cell, in a situation where it is not normally hæmatophagic, becomes nevertheless stimulated to ingest blood elements, it is to be expected that digestion will be fairly complete and successful. We should expect, in short, something comparable to platelet-granules and platelet-cytoplasm to be produced.¹ And, as the former, I would explain the "*Rickettsia*"-bodies which have been found up to the present.

These products of digestion by the endothelial cells may not be identical with those normally produced in the spleen and bone-marrow by the customary macrophages; indeed, in one important respect it seems to me there may be an intrinsic difference, to which I refer below. In this connection, I am reminded that when Captain Horne, I.M.S., and I were studying blood-smears from Egyptian typhus and relapsing-fever patients at Suez, we came across, on two or three occasions, rounded cytoplasmic masses containing no nucleus, but numerous dispersed, red-stained granules; these bodies perplexed us greatly. We came to the conclusion that they occurred only in the blood of typhus cases, because we never found a relapsing-fever spirochæte in smears in which these bodies were present. For a short time we queried the possibility of their being some "parasite"; but even then we came ultimately to the conclusion that these cytoplasmic masses (which were much larger than any individual platelet) had been abstracted from cells of large mononuclear type. In the light of my recent work, I have no doubt now that these masses represented platelet-cytoplasm, formed in a local endothelial lesion.

¹ Whether there is much or little production of actual "platelets" will depend (inversely) on the extent to which multiplication as a whole (proliferation) of particular cells takes place.

On this view the number of platelets, or platelet-like bodies, may be expected to be increased to some extent in typhus. I am not aware of any paper giving actual platelet counts; the only reference I have is to a paper by Cazeneuve (*Bull. Soc. Path. exot.*, 13, 1920), in which the author remarks that "leur nombre était considérablement augmenté."

Readers interested in this subject will know that there is by no means general agreement on the part of workers as to the cause of typhus and typhus-like diseases. Even as regards the Rickettsias, Stevenson and Balfour, in their paper published only last July, "confess to having doubt as to their exact nature, let alone their pathological significance"; and they are not the only ones. Now, as regards the Rickettsias found in insects, I do not wish to say anything about these; and I leave the question of their nature and significance entirely to my confrères at the Lister Institute, who have done the most valuable work in this connexion. All that I have been concerned to do here is to give my reasons for considering that the alleged parasites of this type hitherto described from cases of the above diseases are much more likely to be products of hæmatophagy. And my point is that, if such is the case, *nothing* has been so far detected in the human body which can be regarded with any degree of certainty as the causative organism. In other words, in typhus as in the "Chlamydozoan" diseases, we are apparently still as far as ever from unmasking the elusive micro-organism therewith associated.

HÆMATOPHAGY AS A POSSIBLE CAUSE OF DISEASE.

I know quite well that in all these cases it can be said that there is, nevertheless, some ultra-microscopic, practically invisible organism, or organismal phase, present. But I will ask readers to dismiss from their minds for a moment all thought of a living, specific micro-organism in this connexion, and consider whether there is not another possibility with regard to the virus.

The various formations, bodies, etc., to which reference has been made, all occur in cases where, apart from the question of themselves, no microbic cause has yet been definitely ascertained. On the other hand, so far as I am aware, with one exception,¹ no corresponding bodies have been described in known microbic diseases, at any rate, as of general occurrence and on any important scale. Whereas these formations, if not in all cases absolutely specific, are nevertheless found so constantly as to be diagnostic. In themselves, these bodies are to be regarded as nothing more than the results of an attempt to digest red corpuscles (and sometimes, probably other blood-elements also). But it can hardly be doubted that they signify much more than that; namely, that these formations *stand in some fundamental pathological relation to the disease*. And I venture to think

¹ Namely, the formations which have been described in connexion with syphilitic lesions, by E. H. Ross and others (see also below).

they may nevertheless hold the secret of the ætiology, although they are not parasites.

What is necessarily associated with the production of these bodies? There must be some ferment or enzyme secreted, to begin with, causing the alteration in the composition of the ingested material which becomes manifest. Secondly, there will be certain products of the interaction formed which may be regarded as being of the nature of waste or excretory substances, unusable by the cell. Here, the domain of Bio-chemistry is entered, and it is in the hands of the Bio-chemists rather than in those of the Bacteriologists that I think the elucidation of the cause of these diseases will lie. I am only able to indicate, roughly, the general idea that is in my mind.

In the first place, may not some proteid substance formed as a result of this unusual metabolism be actually harmful to the organism; in other words, itself assist in giving rise to some of the symptoms of the particular disease? The formations themselves, or the contained elements in the case of conspicuous "bodies," or, ultimately, the products of their degeneration and disintegration, may constitute this toxic substance.¹ Alteration or decomposition of organic material (e.g., the albumens, etc.) is known to result in the formation of various substances like the ptomaines, tyrosine, creatine, etc., some of which are toxic. Again, the chemical interaction and its results in one case may differ slightly, but all-sufficiently, from those in another, for instance in the case of an epithelial cell as compared with a nerve cell; just as the toxin produced by one bacterium differs in some remarkable manner from that formed by another. Where the question is one of organic processes by different living cells, both specificity and diversity can be readily understood. On the other hand, the very fact of this unusual ingestion and attempted digestion itself, with its various implications and possible accompaniments (e.g., multiplication, inflammation, necrosis) may suffice to explain the symptoms in certain cases.

Now, what is to be regarded as the virus, the ætiological agent which starts hæmatophagy and the disease? I suggest that this is some *ferment*,

¹ It is important to note that, certainly in one case, these formations are *not* toxic; i.e., the result of unsuccessful digestion is not injurious. I refer, of course, to the Kurloff-bodies in the guinea-pig's lymphocytes. Now, as indicated earlier, the contained elements here are morphologically different from the "inner formations" in, for instance, the Negri-bodies; they tend very frequently to be in the form of curved rods and wavy threads. And in the case of the corresponding bodies found in syphilitic lesions, and regarded by some authors as associated with *Treponema pallidum*, these, too, are very similar to the Kurloff-bodies, often showing wavy threads, etc.; here, also, they appear to be, at any rate frequently, in lymphocytes. Hence they, too, may not have any ætiological connexion with the disease (syphilis), although they probably are an abnormal consequence thereof. What all these varied proteid substances are will be a difficult question, I am afraid, even for the bio-chemists to answer.

the ferment produced to attempt the digestion, by the particular type (or types) of cell concerned; this ferment will vary, of course, in different diseases. I would account for the necessary increase in amount of the virus and for its spread and dissemination in the following way. Infection with a minute quantity of the ferment *stimulates fresh cells of the particular type to hæmatophagy and the production of more of this same substance*. Granted the stimulant property, the metabolic processes in connexion with hæmatophagy will supply the "multiplicative" factor. Thus inoculation will lead, after an incubation-period, to the development of the visible signs and symptoms of the disease. More and more of the virus will be produced, until, in a favourable case, antibodies developed in response are sufficient to counteract it.

That this is no merely fanciful view is indicated, I think, by what is known in connexion with the so-called "bactériophage" of d'Herelle, which provides a most instructive analogy. The consensus of best-informed opinion is, I gather, that we have here no actual living micro-organism, but a bacteriolytic ferment ("catalyser"), a minute quantity of which induces the bacteria themselves to produce more of this lytic agent and, as a consequence, to undergo autolysis. Essentially, the process may be regarded as one of *self-digestion*. In the case of the virus of the diseases under consideration, the main difference would be that the ferment induces not self-digestion, but (attempted) *blood-digestion*, at any rate primarily.

Consider, in this connexion, the closely allied cases of vaccinia and variola. The upholders of the parasitic nature of Guarnieri's bodies have maintained that one principal cause of the difference between the two diseases is due to the fact that in the former case the parasites remain intracytoplasmic, and in the latter they become intranuclear as well. We have to deal, however, not with parasites, but with ingested blood-elements in process of alteration. And I can easily understand that, in response to a strong stimulus, the ingested material may become actually included within the nucleus. If readers will turn back to figs. 7 and 8, it will be seen how, in the course of normal hæmatophagy by the megakaryocytes, the ingested food may be completely surrounded by the nucleus; actual penetration is only a stage further (cf., indeed, fig. 4*b*, where corpuscular fragments are actually inside the nucleus of a large mononuclear). And where the alteration takes place within the nucleus, under abnormal conditions, the substances produced, including the ferment, may well be more concentrated and potent.

The dissemination of this ferment-virus will be brought about by the breaking down of the digesting cells; indeed, it may be discharged even before this takes place. I have above indicated, in the case of the Negri-bodies, that this ferment can probably be poured out of the nerve-cells. Its transmission by means of dejecta, etc., can be readily effected.

As regards typhus in this connexion, in a recent review by Banus (*Intern. Journ. Public Health*, 1, November, 1920, p. 351), reference is made

to certain work by Kusama, which may be, I think, of the greatest significance. This author claims that the virus in the blood is attached to the blood-platelets; he isolates the virus from the platelets by grinding them with the plasma, in which the virus is then found after centrifugalization. Even then the virus does not pass through the filters. *This is precisely the situation (in the blood) in which one would expect the virus to be most concentrated, on the view here put forward.* I mentioned above that although, as cells of endothelial type are concerned, the digestion might be expected to be fairly complete, with production of platelet-cytoplasm; nevertheless, it would probably not be completely normal, as these particular cells are not those which customarily exercise the function of macrophages (they are specialized to form the walls of capillaries, etc.). Therefore, in this metabolism, both an unusual ferment and (perhaps) slightly different by-products will be concerned. This ferment, the virus, will be *in the platelet-like bodies resulting from the platelet-cytoplasm produced by these endothelial cells in the capillary lesions*, but it will probably not be in the normal platelets, normally formed by the customary macrophages.

Many will still hold that there is some invisible microbe, either inside the platelet or fortuitously attached to it; others will say that what are considered to be platelet-granules are really Rickettsias; but I think it is most likely that the bodies regarded as *Rickettsia* are in reality, elements essentially comparable in origin with platelet grains and granules, and that the platelet-cytoplasm and platelet-like bodies containing them, differ chemically in some most important respect, which constitutes the active principle of the virus.

There is a saying that one is sometimes in danger of missing the wood for the trees. On the other hand, it may be said that I have not paid enough attention to the trees, because of the wood. Up to the present, I have only been able to study three in any detail, and there are probably many trees in this particular wood; to know them all completely will mean much further work, by many workers. *But there is a wood*—a fascinating wood—and as soon as I realized this I became most desirous of gaining some idea of it as a whole, its general character and apparent extent. Viewing my wood from afar, I believe that I have caught a glimpse, indistinct though this be, of its nature and configuration. And from this distance, it seems to me there stands within its confines a giant of the forest, towering above the other trees and spreading wide its gnarled and twisted branches; a thing indeed of menacing aspect. This gloomy, malign form, I am now endeavouring to approach more nearly, intent on learning whether, of a truth, it also is included in the wood called hæmatophagy.

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EXPLANATION OF FIGURES.

FIG. 1.—Platelets, and a lymphocyte for comparison. The platelet-granules are distinct in character from those of the lymphocyte. (Smear, Giemsa; $\times 1,600$.)

FIG. 2.—Platelets, showing the appearance according to the amount of extraction of the black stain (see text). Corpuscles, polymorph, and lymphocyte for comparison. (Film, iron-hæmatox. + eosin; $\times 1,600$.)

FIG. 3.—A field in a de hæmoglobinized smear. (Iron-hæmatox., undifferentiated; $\times 1,600$.)

FIG. 4.—(a) Nucleated reds, red corpuscles and fragment of same. The other figures show the ingestion of corpuscles and free nuclei by the large mononuclears, and the formation of platelet-granules in the cytoplasm, as a result of digestion; (b) cell containing several unaltered (recently ingested) corpuscles, or portions of corpuscles, each separate from the others; (c) cell containing an ingested nucleus and also a fragment of a corpuscle; (d) portions of abstricted platelet-cytoplasm, of varying size, down to that of a discrete platelet (e). (Smear, Giemsa; $\times 1,600$.)

FIG. 5.—Nucleated reds (normoblasts) (a), and free nucleus of the same (b). Megakaryocytes to show cell-history, beginning as cell of large mononuclear or "transitional" type; (c) large amœboid form, containing two lymphocyte-nuclei; (d) platelet-cytoplasm, in different stages, being thrown off; (e) a hungry megakaryocyte, containing several recently ingested, immature red-cell nuclei; (f) ultimate disintegration of a megakaryocyte. (Film; $\times 1,600$.)

FIG. 6.—Megakaryocyte (medium size) containing an ingested corpuscle. (Film, Giemsa; $\times 1,000$.)

FIG. 7.—Bone-marrow of a purpuric guinea-pig, showing two large megakaryocytes. The one to the right contains a huge "vacuole," with nine included eosinophil polymorphs; and another is present in the cytoplasm below. That to the left contains a mass of ingested corpuscles (indicated by an arrow), the upper part being denser than the lower; the dark body to the right of the mass is the nucleus of the cell. (Dr. Ledingham's section; $\times 500$.)

FIG. 8.—Bone-marrow of purpuric guinea-pig, also showing two megakaryocytes. The upper one contains four eosinophils inside the nuclear ring; the lower one possesses a huge ring-like nuclear complex, and in its centre are two recently ingested red corpuscles (indicated by an arrow). Two eosinophils are seen in the cytoplasm just above. (Dr. Ledingham's section; $\times 500$.)

FIG. 9 (on same plate as fig. 6).—Megakaryocyte, showing platelet-cytoplasm and aggregations of platelet-granules at the periphery; the clearer area, to the left of the nucleus, indicates recently ingested corpuscles. (Smear; $\times 800$.)

FIG. 10.—Kurloff-body (medium size) in lymphocyte. (Smear; $\times 1,600$.)

FIG. 11.—Red corpuscles (*a*); large mononuclears containing ingested corpuscles (*b*), one of which shows platelet-cytoplasm fraying out at the right-hand side; (*K*) various conditions of the Kurloff bodies in lymphocytes; (*f.K.*) two free Kurloff bodies. (Film; $\times 1,600$.)

FIG. 12.—A, Large Kurloff body, in a lymphocyte about to divide, showing the pale inclusions in the rose-coloured contents of the "vacuolè"; B, Kurloff body showing distinct granulations in addition to the rose-coloured mass. (A, film, Giemsa; B, smear (see text), Giemsa; $\times 1,600$.)

FIG. 13.—Stages in the transformation of corpuscles into Negri bodies. (*a*) Portion of a field; this shows a long capillary with an endothelial nucleus near each end, and containing (close to the right-hand nucleus) two corpuscles in contact; other unaltered corpuscles (*c*) are scattered about, also neuroglial nuclei (*neur.*); a short, rather curved capillary segment, rounded and closed at each end, in which the contained corpuscles are polychromatophilic; two Negri bodies (*N*), in an early stage, are also seen. In the right-hand half of the figure are various stages in the alteration of corpuscles or corpuscular masses; (*b*) a portion of a corpuscle still quite unaltered is contained in this mass undergoing alteration (at the lower left-hand corner); (*d*) fully-formed Negri bodies; (*e*) a narrow capillary segment containing a fully developed, cigar-shaped Negri body and one or two unaltered corpuscles. (Smear, Giemsa; all $\times 800$ excepting (*d*), $\times 1,600$.)

FIG. 14.—Negri bodies in sections. A, Field from a section stained by Mann's method; B, small portion of a field stained by iron-hæmatox. + eosin; (*c*) unaltered corpuscles, often crinkled; (*N*) Negri bodies, or altered corpuscles becoming such (in B); (*n*) nucleoli of nerve-cell nuclei; (*neur.*) neuroglial nuclei. (A $\times 800$; B $\times 1,600$.)



FIG. 1.

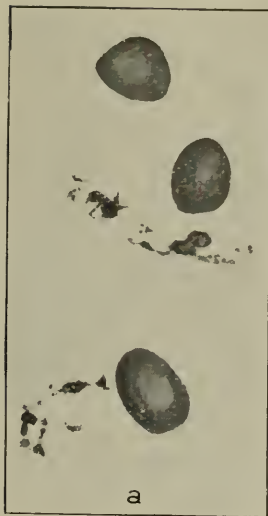


FIG. 2.

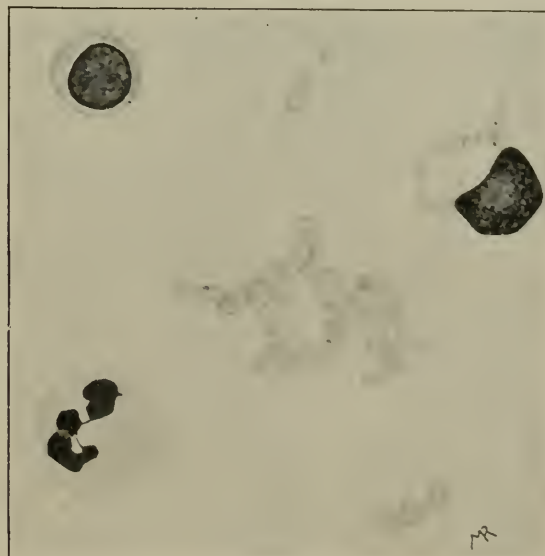


FIG. 3.

To illustrate "An Introduction to the Study of Hæmatophagy," by H. M. Woodcock, D.Sc.Lond.



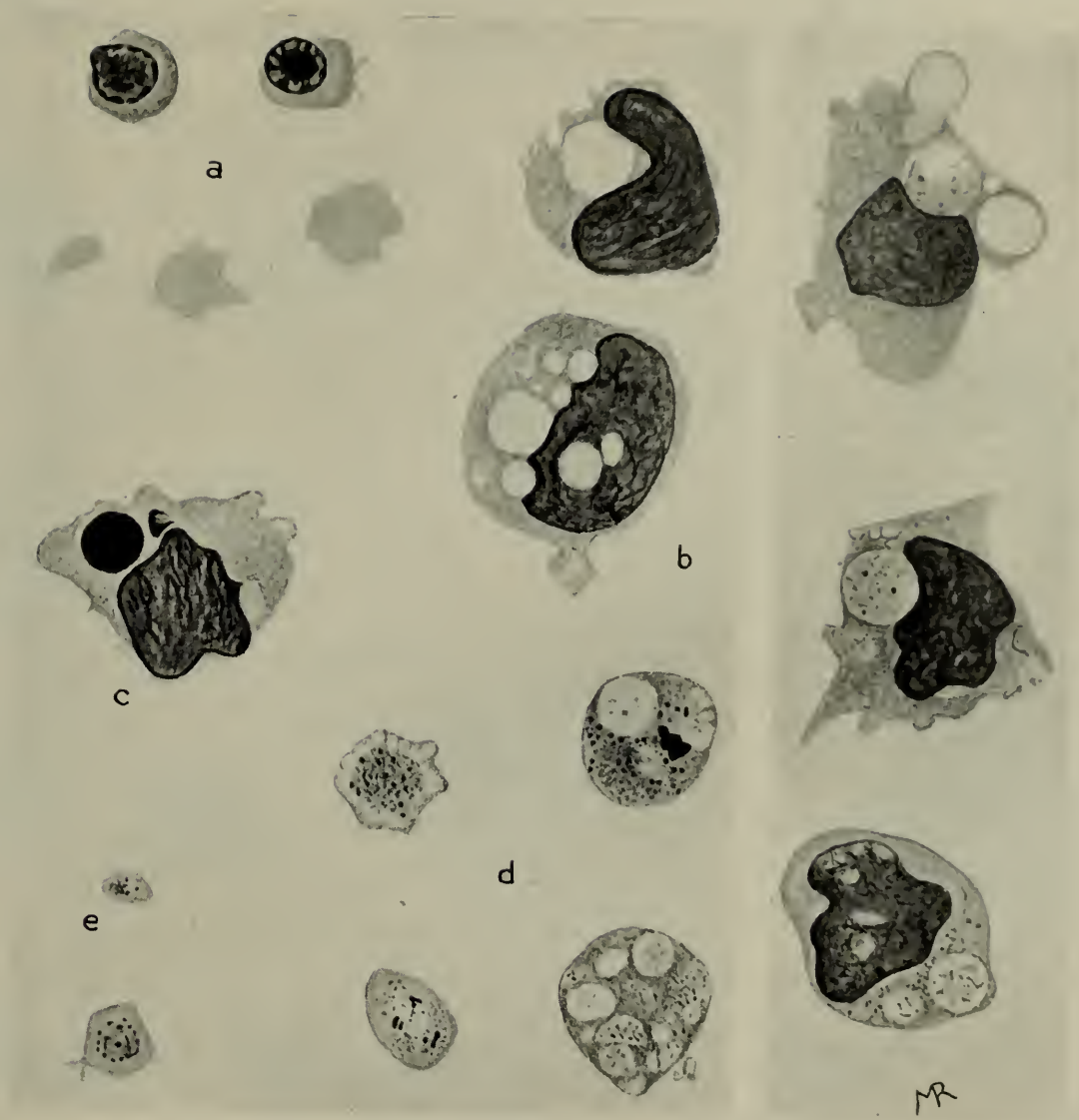


FIG. 4.

To illustrate "An Introduction to the Study of Hæmatophagy," by H. M. Woodcock, D.Sc.Lond.



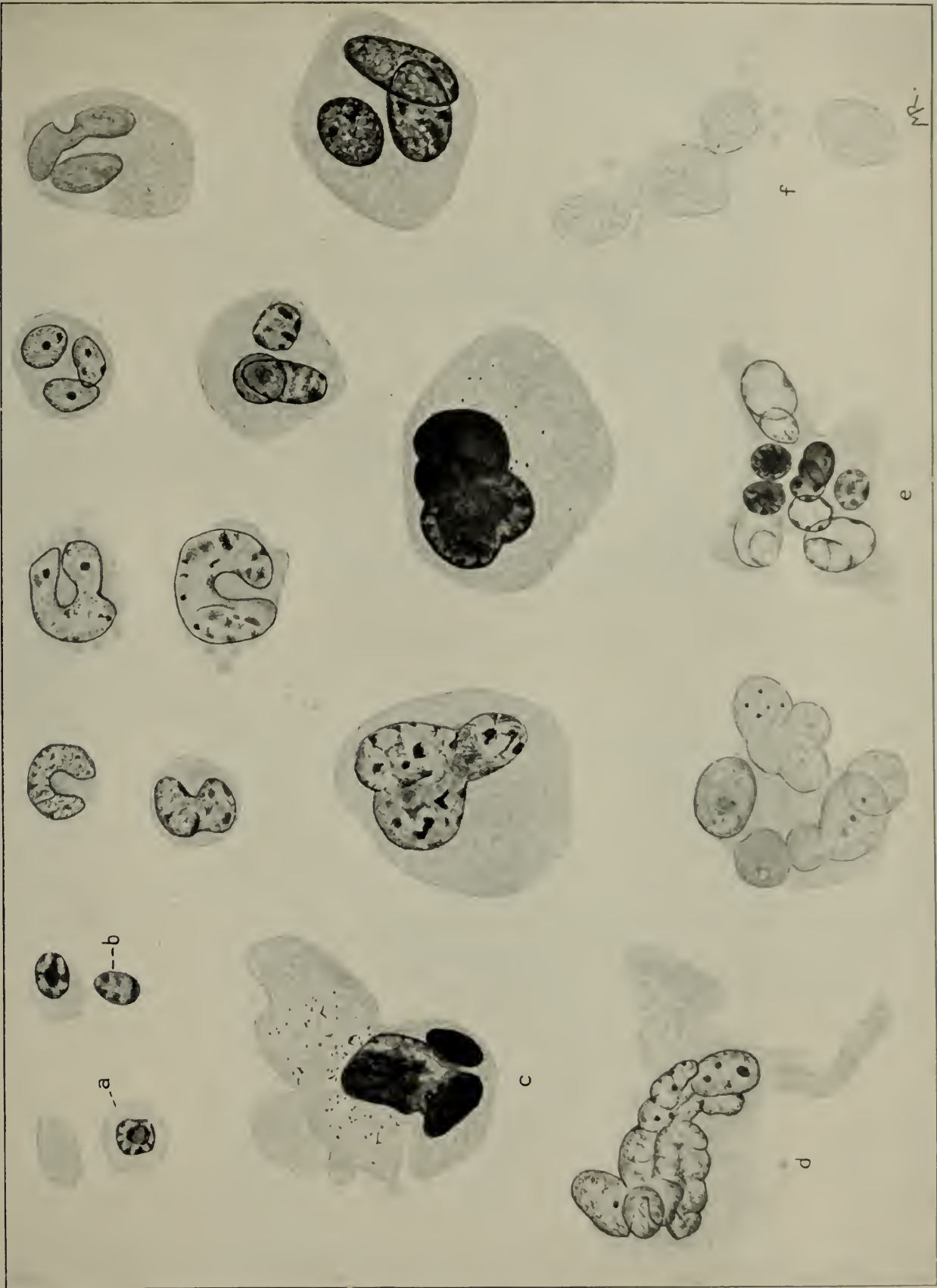


FIG. 5.

To illustrate "An Introduction to the Study of Hæmatophagy," by H. M. Woodcock, D.Sc.Lond.



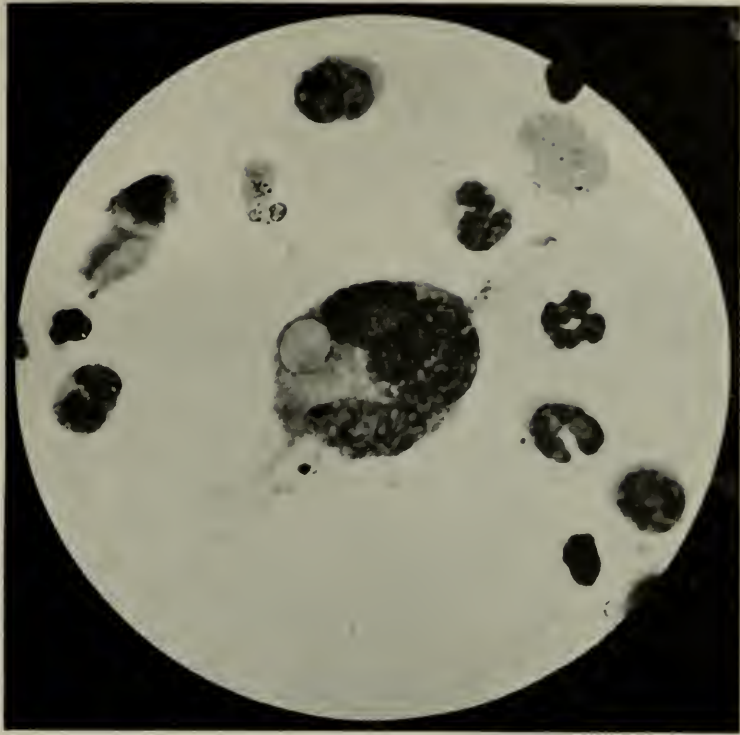


FIG. 6.

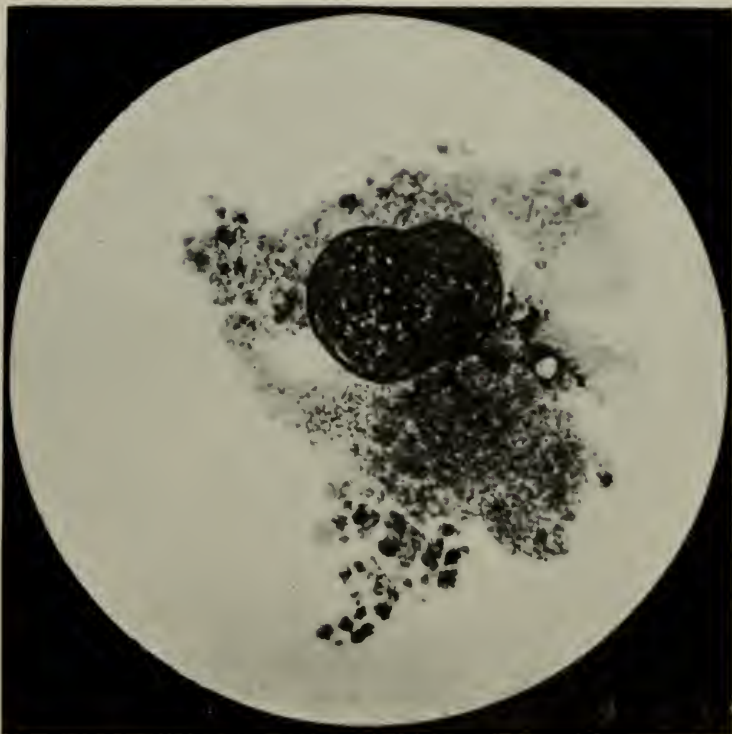


FIG. 9.

To illustrate "An Introduction to the Study of Hæmatophagy," by H. M. Woodcock, D.Sc.Lond.



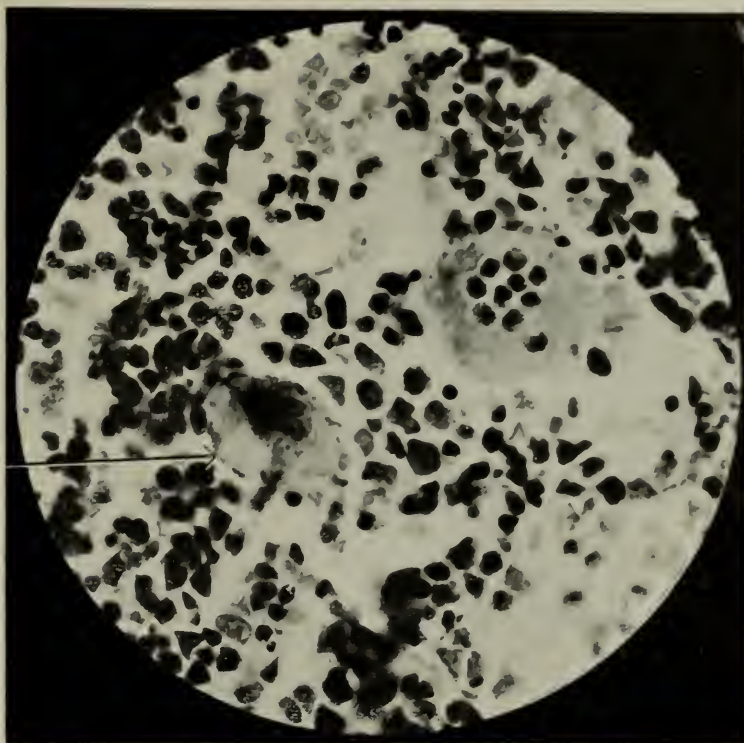


FIG. 7

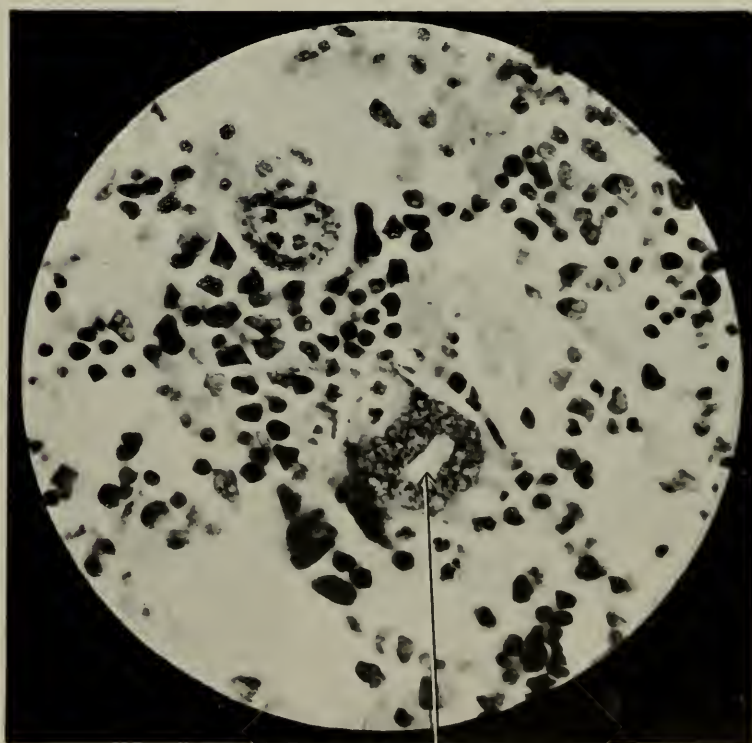


FIG. 8.

To illustrate "An Introduction to the Study of Hematophagy," by H. M. Woodcock, D.Sc.Lond.



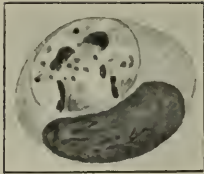


FIG. 10.

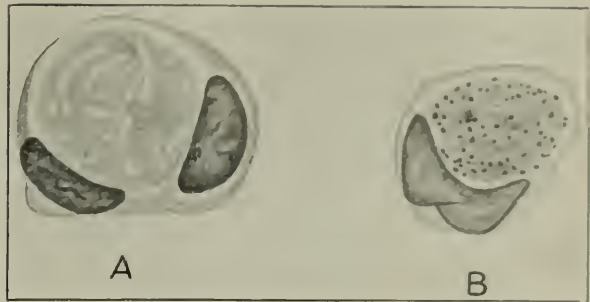


FIG. 12.

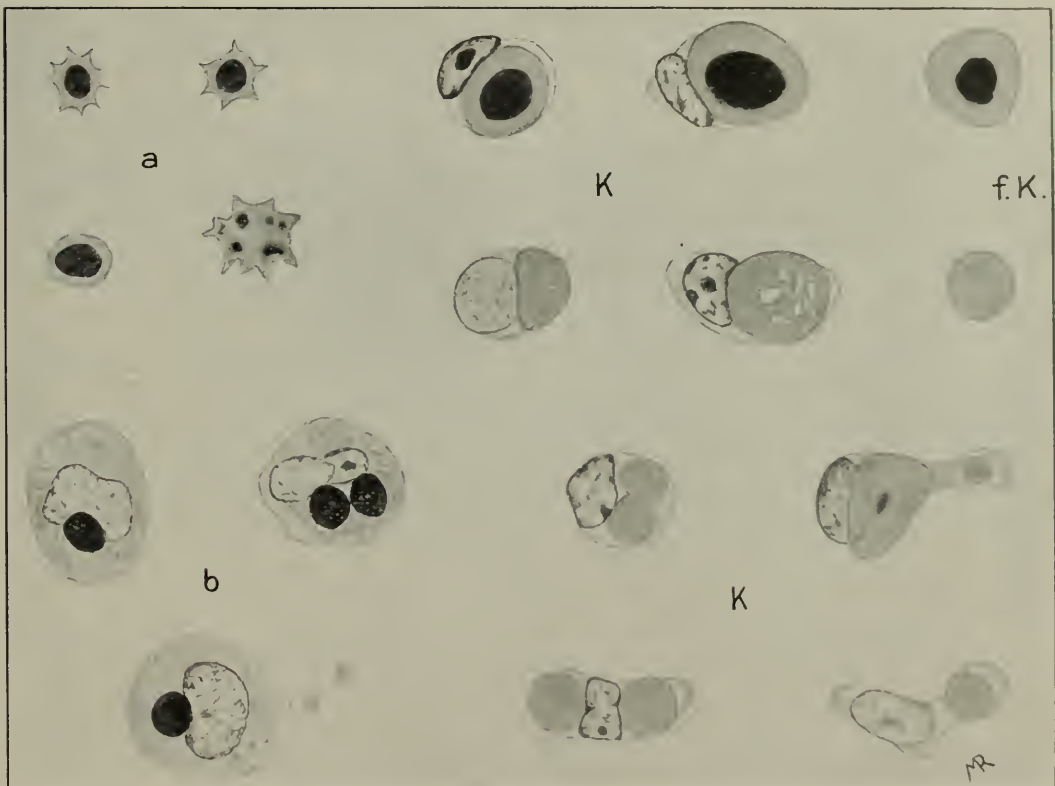


FIG. 11.

To illustrate "An Introduction to the Study of Hæmatophagy," by H. M. Woodcock, D.Sc.Lond.



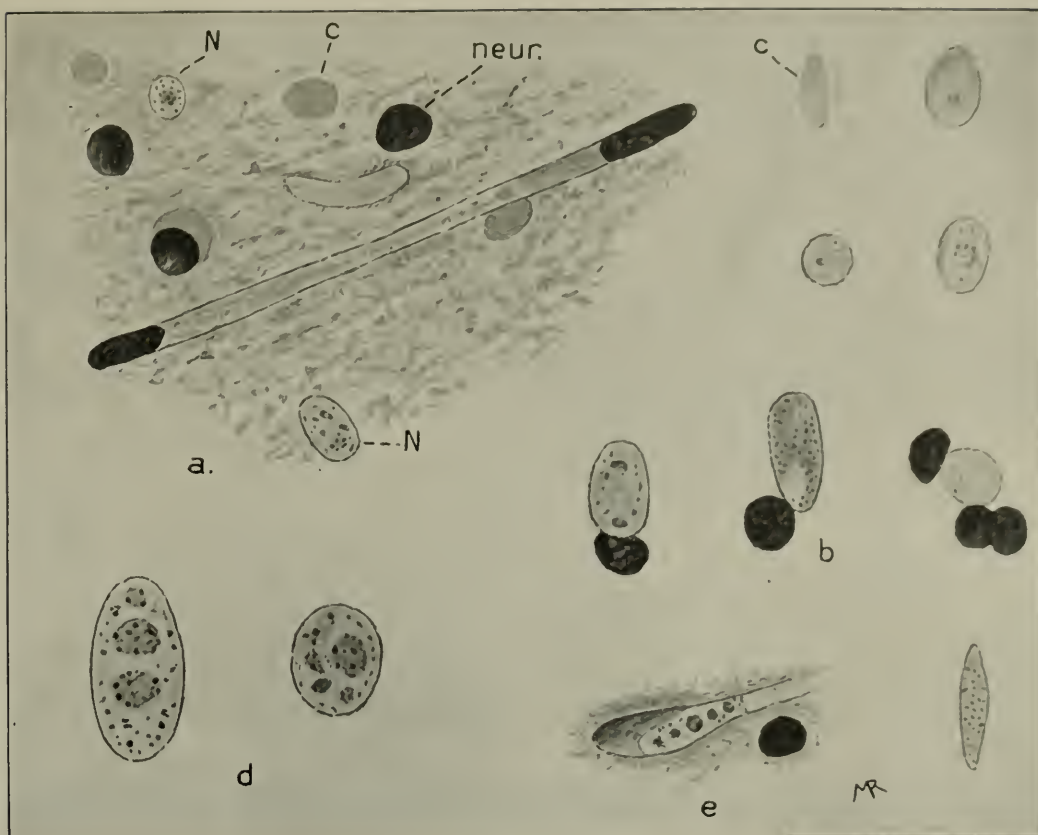


FIG. 13.

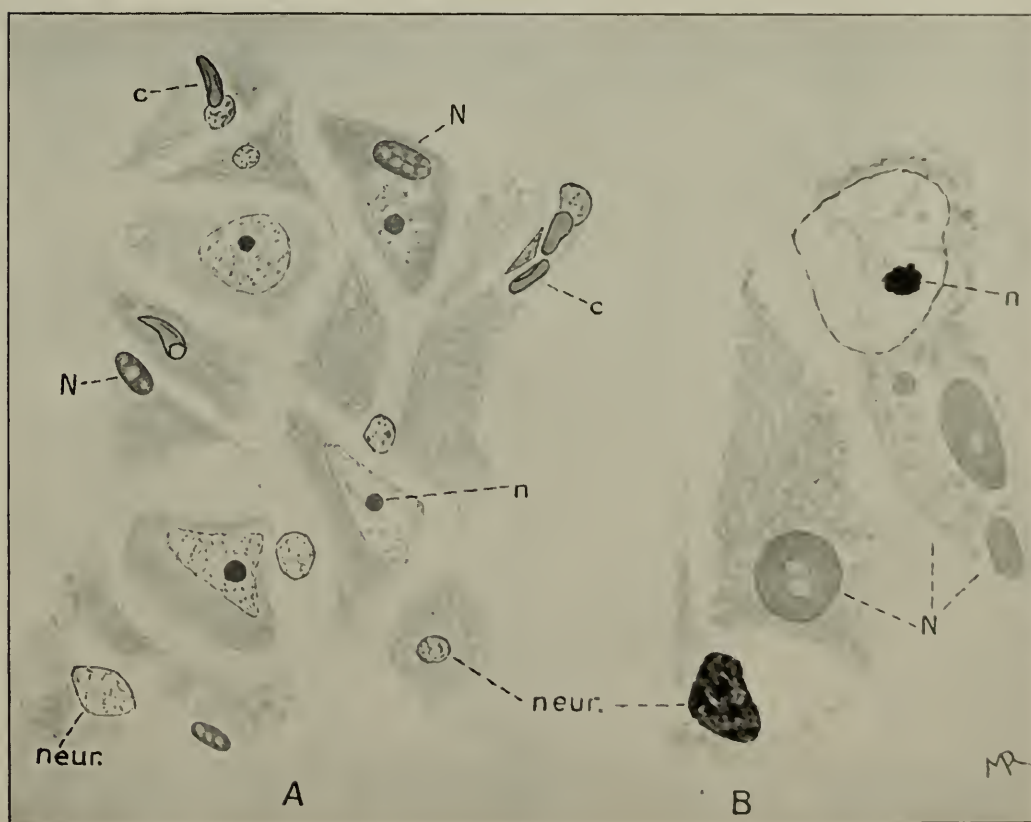


FIG. 14.

To illustrate "An Introduction to the Study of Hæmatophagy," by H. M. Woodcock, D.Sc.Lond.

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XXI. VITAMIN REQUIREMENTS OF *DROSOPHILA*. I. VITAMINS B AND C.

BY ARTHUR WILLIAM BACOT AND ARTHUR HARDEN.

From the Departments of Entomology and Biochemistry, Lister Institute.

(Received January 30th, 1922.)

LOEB [1915] ascertained the fact that flies of the genus *Drosophila* could be reared on a culture medium containing as mineral constituents only K, Mg, PO₄ and SO₄ ions. In these experiments micro-organisms were not excluded, and the growing larvae undoubtedly lived at the expense of these. Loeb also found that when Na was substituted for K no growth took place and concluded that K was essential for the development of the insect. It is however possible that the absence of K stopped the growth of the micro-organisms and thus deprived the larvae of nitrogen in an available form. Loeb's experiments therefore only prove that the mineral constituents named above are adequate, but not that they are essential.

Subsequently Loeb and Northrop [1916] succeeded in sterilising the eggs of *Drosophila* by exposure for 6–7 minutes to 0.1 % aqueous HgCl₂ or a saturated alcoholic solution of the same salt and found that the larvae hatched out from the few eggs which survived the disinfection were sterile. They ascertained that these sterile larvae could not be reared on sterile media consisting only of purified proteins, carbohydrates, fats and salts, but that when yeast killed by heat was added, normal development occurred. Alcoholic extracts of yeast were however inactive. Northrop [1917] pursued the subject further and found that the addition of caseinogen and similar substances along with cane sugar to yeast greatly increased the number of flies which could be reared at the expense of a definite amount of yeast and concluded that they acted as foodstuffs when properly supplemented by yeast. Similar results were obtained by the addition of kidney, liver and pancreas from the dog, but many other organs of the dog were inactive. The author was inclined to interpret these results in the sense that yeast supplies one or more accessory substances, necessary for the growth of the organism.

The question was again investigated by Baumberger [1919] who found that the pupae and eggs of flies reared in presence of a pure culture of yeast could readily be sterilised by immersion for 20 minutes in 85 % alcohol. The sterile larvae obtained from such eggs could easily be reared in sterile culture media provided the latter contained killed yeast or nucleo-protein prepared from yeast. Baumberger came to the conclusion that the essential requisite

was a sufficient concentration of yeast nucleo-protein and that accessory food factors were not concerned.

The following experiments were instituted with the object of ascertaining more definitely whether any of the recognised vitamins are essential for the nutrition of these flies. The subject is of general interest as regards the extent to which the need for accessory food factors is distributed in the animal kingdom. Special interest attaches to the possibility of introducing a simplified technique by which results could be obtained more rapidly and economically than by the tedious and costly feeding experiments which are at present necessary.

TECHNIQUE OF THE EXPERIMENTS.

It was soon found that the eggs could be sterilised much more readily and certainly than the pupae and this method was always employed.

Following Baumberger's instructions the flies were reared in banana-agar (approximately equal parts of mashed banana and 1.5 % water-agar) which had been autoclaved and subsequently inoculated with yeast (*S. cerevisiae*). After a few sub-cultures on this medium the flies were found to be free from bacteria, both anaerobic and aerobic, and the pure stock was readily maintained by occasionally passing a few flies into a fresh banana-agar-yeast tube.

When eggs were desired for experimental purposes, freshly emerged flies were transferred to a special apparatus. This consisted of an inverted wide necked bottle, standing in a Petri dish and having a strip of moistened filter paper on the wall. A small circular glass dish with vertical walls was also placed on the Petri dish, so as to be covered by the inverted bottle. The walls of this dish were lined with hardened filter paper moistened with water. The bottle was in turn covered by an inverted cylindrical tinned iron can and the whole apparatus was autoclaved at 120° for half-an-hour. A few cc. of a 24-48 hour culture of yeast in yeast extract containing glucose were then placed in the small dish, the flies introduced into the inverted bottle and the whole apparatus placed in an incubator at 30°. Under these conditions the eggs were laid within 24-48 hours almost exclusively on the hardened filter paper lining the small circular glass dish containing the yeast culture. The strips of paper were removed, placed on a sterile slide under a dissecting microscope and the eggs picked off singly by means of sterilised dissecting needles and placed in 85 % alcohol as directed by Baumberger. They were then divided up into collections of the number required for each tube by means of a sterile pipette provided with a rubber teat, and were finally, after half-an-hour's exposure to the alcohol, sucked up into the pipette and delivered into the appropriate tube of medium. It was found most convenient to place 20 eggs in each test tube (6" × $\frac{3}{4}$ ") containing 5-5.5 cc. of medium in the form of an agar slope.

The agar jelly in the medium tube must be so soft that the larvae can readily penetrate it in their search for food. As a rule 3 cc. of 1½ % agar

were added to 2 cc. of medium and the whole then steamed for 20 minutes on three successive days, and cooled in the form of slopes.

The inoculated tubes were incubated at 30° and examined daily, note being made of the number hatched, the general progress and the time of appearance of pupae and flies.

At the close of each experiment the tubes were tested for sterility by making cultures on beef broth-peptone-agar and incubating at 37°. Contamination by yeast usually showed itself in the experimental tube, but was when necessary tested for by culture on beer wort agar at 25°.

DIET.

A basal diet consisting of

Caseinogen (purified)	0.15 g. per tube
Starch and salts ¹ (94 % starch and 6 % salts)					0.05 g. „
Cane sugar	0.1 g. „

was employed, to which various additions of butter-fat (vitamin *A*) yeast-extract (vitamin *B*) and lemon juice freed from citric acid (vitamin *C*) were made as required, 2 cc. of liquid in all being added, and then 3 cc. of 1½ % agar. The caseinogen had been extracted with alcohol and light petroleum or in some cases heated at 120° for many hours and repeatedly stirred.

RESULTS.

The experiments were directed in the first instance to ascertaining which, if any, of the three recognised accessory food factors are necessary for the growth and metamorphoses of these insects.

The results, which are summarised below, showed definitely that:

(1) in the presence of butter-fat and yeast extract (vitamins *A* and *B*) growth occurred irrespective of the presence or absence of 1 cc. of lemon juice (vitamin *C*);

(2) in the presence of butter-fat (vitamin *A*) growth occurred in presence of 1 cc. of yeast extract (vitamin *B*), but not in its absence, this result being independent of the presence or absence of lemon juice;

(3) in the presence of 1 cc. of yeast extract (vitamin *B*), with or without 1 cc. of lemon juice (vitamin *C*), growth occurred in the presence of two drops of butter-fat, but not in its absence.

In the following summary only results of experiments in which the tubes remained sterile are quoted.

	Additions to basal diet			Tubes inoculated	Eggs hatched	Pupae formed	Flies emerged
	Butter-fat	Yeast extract	Lemon juice				
1	—	+	+	10	100	0	0
2	+	+	—	11	115	92	63
3	+	—	+	10	57	0	0
4	+	+	+	10	68	48	42
5	—	+	—	2	19	0	0
6	—	—	—	2	16	0	0

¹ As used in this laboratory for rats.

Control experiments were carried out to ascertain whether the yeast extract alone was capable of supplying sufficient nitrogen for the growth of the larvae, but with entirely negative results. This was done by omitting the caseinogen from mixtures 2 and 4, which gave positive results in its presence. Even the addition of 2 cc. of the yeast extract gave entirely negative results in the absence of the caseinogen.

Other sources of vitamin B.

As flies of this group are specially associated with yeast, which probably forms the chief food of the larvae in their natural condition, it was thought that in order to prove the necessity for vitamin *B*, it would be essential to show that it could be supplied from other sources.

The first experiments were made with milk, but without success. Recourse was then had to wheat germ, the alcoholic (80 %) extract of which is known to contain a considerable amount of vitamin *B*.

50 g. of wheat germ were heated on the water-bath for one hour with 200 cc. of 80 % alcohol, pressed out and the clear liquid evaporated to dryness at 50°. The residue was shaken up with 50 cc. of water and filtered, the clear filtrate being used. This contained 10.47 g. total solids, 0.21 g. mineral matter, and 0.196 g. N per 100 cc.

Tubes were made up with 2, 1 and 0.5 cc. of this extract in addition to the usual basal diet of caseinogen, starch, salts, cane sugar and butter-fat. Some difficulty was experienced in adjusting the stiffness of the agar jelly, but finally 0.5 cc. H₂O and 3 cc. of 1.25 % agar were added to each tube, so that the total volume was 5.5 cc. as shown below.

	Caseinogen, starch and salts g.	Cane sugar g.	Butter- fat	Wheat germ extract cc.	H ₂ O cc.	1.25 % agar cc.
7	0.2	0.1	2 drops	2	0.5	3
8	0.2	0.1	„	1	1.5	3
9	0.2	0.1	„	0.5	2.0	3
10	Starch and salts 0.05	0.1	„	2	0.5	3

The following are the summarised results of three quite independent and consistent experiments:

	Wheat germ extract	Eggs known to have hatched	Pupae	Flies
11	2	34	18	9
12	1	56	24	13
13	0.5	39	0	0
14	2 cc. as sole source of N	9	0	0

The experiments were somewhat hindered by the hot weather prevailing at the time, which caused unduly rapid drying of the tubes, but they show quite conclusively that, like yeast extract, wheat germ extract is capable of supplying some factor necessary for the growth and metamorphosis of the fly.

It is at the same time obvious that the wheat germ extract (1 cc. of which corresponds with 1 g. of wheat germ) is much less efficacious than the yeast

extract (1 cc. of which corresponds with 0.5 g. yeast) and this is in accordance with what is known of the vitamin *B* content of these two materials, 1 g. of yeast being approximately equivalent to 3.6 of wheat germ.

Taken together the experiments with yeast extract and wheat germ extract leave little doubt that these insects require vitamin *B* for their development, thus confirming Northrop's view. Experiments are in progress on the amount of yeast extract required and the effect of varying concentrations on the duration of the period of development.

THE FAT-SOLUBLE FACTOR (VITAMIN *A*).

The striking fact that in presence of 1 cc. yeast extract growth occurred in the presence of two drops of clarified butter-fat, but not in its absence, may be taken to show either that a fat of some kind is necessary or that the fat-soluble factor is required or that both are required. To decide between these possibilities it is necessary to ascertain whether or not growth occurs in presence of a fat devoid of vitamin *A*. Attempts of this kind are being made, but no definite result has yet been obtained. The preliminary results however indicate that the fly is certainly able to develop in the presence of very small amounts of the vitamin if not in its entire absence.

SUMMARY.

The complete development of *Drosophila* requires the presence of vitamin *B* but not of vitamin *C*.

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N^o 2

Observations

ON THE

INFLUENCE OF FOODS RICH IN ACCESSORY FACTORS IN STIMULATING DEVELOP- MENT IN BACKWARD CHILDREN.*

BY

HARRIETTE CHICK, AND ELSIE J. DALYELL,

D.SC.LOND.,

M.B.SYDNEY,

LISTER INSTITUTE, LONDON.

BEIT MEMORIAL FELLOW.

(From the Landes Zentral Kinderheim, Vienna.)

THE children described in this paper were in one ward of a large foundling hospital in Vienna, where we were permitted to work by the courtesy of the director, Dr. Gustav Riether. They varied in age from 1 to 3 years, and in growth, body weight, activity, and general development each was considerably below the normal standard for age and had been so for many months previously. The backward condition was in no way associated with existing illness, and the observations made on the group were of interest by demonstrating each child's approach to normal standard when dietetic changes were introduced.

In this institution there is an effective system of recording the body weight, diet, development, and medical history of every child, and it was therefore possible to trace the progress made since admission in the first weeks of life. The hospital is established in spacious modern buildings, and has a small staff of trained nurses, who supervise the wards. The care of children is undertaken by mothers, admitted with their infants from the obstetric clinics, who remain three to four months, in order to nurse their own babies and to assist in the care of those without mothers. As frequently happens in large institutions, the life of mothers and children is rather confined, and, owing to shortage of fuel, overcrowding could not be avoided in the reduced number of pavilions it was possible to heat.

Careful study of the past diets of the special group of children showed that after the three first months of life the calorie value of their dietary was usually adequate when breast milk had been supplemented by artificial

* Report to the Accessory Food Factors Committee appointed jointly by the Lister Institute and Medical Research Council.

food; but the types of food used suggested that defective growth might be due to qualitative deficiencies in the diet, from shortage of fats and of fresh elements. The frequent occurrence of infantile scurvy (Barlow's disease) in the institution and the prevalence of rickets pointed in the same direction.

The diets of the children consisted of diluted cow's milk, to which was added sugar, cereals, or proprietary infants' foods. The amount of vitamins in the food was therefore reduced in proportion to the amount of this dilution, and the same was true of the fat content. The antiscorbutic value of the milk food was further reduced, owing to the time that elapsed before it was consumed. It was reckoned that all milk was three to four days old when it was consumed in the wards. There is also little doubt that during the warm months the milk was heated at least once before delivery. All the food for some hundreds of infants was prepared in a central milk kitchen by standardized methods, which were studied in detail. The kitchen arrangements were admirable and there was no overcooking, but the irregularity of the milk delivery and the necessity for preparing large quantities at once, caused delay in the whole process of preparation. The food was prepared according to standard receipts under the control of one sister who had been in charge of the milk kitchen for over ten years.

By the courtesy of the director and with the co-operation of the physician in charge of the ward, dietetic treatment of a group of children was undertaken from December, 1919, to June, 1920. The main diet was still supplied from the central kitchen, but addition was made of (1) antiscorbutic material to supply this accessory factor, and (2) fats rich in the fat-soluble accessory factor.

Condition of the Children before Treatment.

In this communication nine children are specially selected for study; their ages ranged from 12 to 31 months. A short survey of their condition when first seen is set out in the accompanying table. All were under the normal weight for their age and in most cases the deficiency amounted to 25 or 30 per cent. The delay in development was more striking than the low body weight.

Four of the nine children could not sit up without support. Case 7 at 21 months lay inert, and if a limb were raised it fell back helplessly. Case 9, 31 months old, could not hold its head up for more than a few minutes and took no notice of its surroundings or of being handled. The absence of any spontaneous activity or movement among the children was a very striking feature. They showed little signs of awakened intelligence, they had no desire to play, and the older children made no attempts at speech.

With two exceptions, weight at birth was normal; Case 1 was a premature baby weighing 2,000 grams, and Case 8 was a twin and weighed only 1,600 grams. All had

had some amount of breast feeding in the early months of life, and one child had had a complete diet of breast milk until 7 months old; most of the children, however, had made little progress at first, in spite of the breast feeding. It should be noted that the dates of their birth—1918 or early 1919—coincided with a period of great food deprivation in Vienna, and it is therefore probable that the mothers were imperfectly nourished during both pregnancy and lactation. In hospital, however, there was no serious shortage of calories in the diets after the breast feeding was supplemented or replaced by artificial food. The supplementary artificial food began from birth in 4 cases, and before $4\frac{1}{2}$ months in 5 cases.

From the data available the calorie value and the proportion of milk fat in the diets were calculated from birth up to the time of the first examination of each child. The energy quotient or ratio of the calories consumed daily to the body weight expressed in kilograms was also calculated. This value was always maintained at the accepted* standard and averaged well over 100; in some cases it reached 150, 180 and even 200 for varying periods of time. The amount of milk fat was low in comparison with that taken by an infant fed on whole milk of good quality, whether human or cow's. The antiscorbutic value of the diet was low for the reasons given above.

Previous Illness.

The past history of the children showed furunculosis and otitis in many cases, but the outstanding fact was the occurrence of definite scurvy once or oftener in every instance except Case 1. Case 8 had suffered from two attacks, at $6\frac{1}{2}$ and 13 months respectively, and Case 9 (31 months old) from three attacks, at 16, 20, and 24 months respectively. These two children were the most backward of the series considering their age. The symptoms of scurvy had consisted in swelling and tenderness of the limbs, especially the thighs, and in haematuria; mouth symptoms were rare.

In the treatment of scurvy in this institution some whole raw milk was given in the diet for a period varying from one or two weeks to some months, the time depending on the rate at which the symptoms subsided, and the amount upon the supply of raw milk available. In some cases raw lemon juice was also given, but no satisfactory method existed for its administration. Recovery was slow and often partial; in Case 8 symptoms persisted over a period of several months. Study of the weight charts of these children convinced us that failure to increase in weight was often associated with deficiency of antiscorbutic material in the diet. In many cases increase in

* Lusk (*Science of Nutrition*, third edition, 1917, p. 404) concludes that 80 calories per kilo of body weight would suffice during the first year of life; Heubner (*Berl. klin. Woch.*, 1901, vol. xxxviii, p. 449) allows 100 for the first three months of life, 90 from three to six months, and 80 afterwards; Holt (*Amer. Journ. Dis. Child.*, 1921, xxi) places the standard at 120 for the first few months and at 100 until the end of the first year.

weight had taken place when extra antiscorbutic was added to the diet, or when raw milk was substituted temporarily for the heated milk food.

Presence of Rickets.

Some symptoms of rickets were present in all children, and in five cases the disease was severe and definite deformity was present. A survey of the whole institution, made in March-June, 1920, showed that some degree of rickets was common at 5 and 6 months of age, and that the disease was practically universal at 9 months—that is, at a period considerably earlier than that at which it is ordinarily supposed to be manifest. In arriving at a conclusion as to the existence of rickets attention was directed to the following points:

1. Condition of anterior fontanelle; presence of cranio-tabes; development of parietal and frontal bossing.
2. Enlargement of costochondral junctions; development and contour of chest.
3. Enlargement of epiphyseal ends of long bones at wrists and ankles; bowing of the tibiae.

Description of Treatment.

Treatment consisted in enriching the diet of these children with (1) the antiscorbutic vitamin and (2) the fat-soluble vitamin in form of animal fat. The diets remained otherwise unchanged except that in some cases food was withdrawn to balance the additional calories given in the form of fat. The energy quotient was in no case increased. The general management of the children remained the same.

The antiscorbutic material was given in the form of raw swede turnip juice, 10 to 20 grams daily; experimental work on scurvy in guinea-pigs had indicated that the raw juice of this vegetable possesses antiscorbutic properties comparable with those of fresh oranges or lemons. The clean cut surface of the raw vegetable was grated on an ordinary kitchen grater and the pulp squeezed in muslin by hand. It was given alone or with the food and was well tolerated in all cases. It was occasionally replaced by orange juice or lemon juice (neutralized with solid calcium carbonate and filtered), but during the winter 1919-1920 these fruits were too scarce and too expensive in Vienna for general use.

The fat-soluble vitamin was given in the form of cod-liver oil and butter. The initial dose was 10 grams of butter daily; in February, 1920, the daily dose of butter was increased to 20 grams for five of the nine children, and to these, in the month of April, 10 grams of cod-liver oil daily were given in addition. Cases 1 and 3 received cod-liver oil only, and Case 2 received an extra ration of 400 c.cm. full milk daily—that is, about 12 grams of milk fat.

The result of these additions to the diet was in every case satisfactory. The children began to put on weight at

a more rapid rate than formerly, and the normal curve was gradually approached during the six months of treatment (see Table). The improvement is specially evident in Cases 8 and 9, where departures from the normal weight for the age, as great as 4 and 4.6 kg., were reduced to 1.2 and 2.4 kg. respectively.

But more striking than the increase in weight was the improvement of the children in general activities. Taking the four children, aged respectively 12, 21, 22, and 31 months, who were unable to sit alone, Case 7 could do so after six weeks, and Cases 1, 8, and 9 after two and a half to three months of treatment. Of seven children who showed no spontaneous movement of the legs when first examined, four (Cases 1, 4, 5, 8) stood within three months, and after six months all the children were beginning to walk. Another sign of satisfactory development was the rapid closing of the fontanelle, which in every case was open before treatment was begun.

Data collected from the past histories and during the period when the children were under observation were set out in charts showing the calorie value and fat content of the diets, together with the weight curves, plotted against the normal. That given by Pfaundler (1916) has been adopted as giving a fair average for normal growth. The past histories proved to be of great interest, especially as showing the influence of scurvy or of a prescorbutic condition upon increase in weight. It would take up too much space to give the detailed description of all the nine children, but four cases (4, 7, 8, and 9) have been selected as being specially instructive. Case 4A, that of a little girl nearly two years old, not, however, treated in the present series, has been added as a striking instance of growth induced by adding antiscorbutic to a deficient diet.

CASE 4. (Chart 1.)

At birth, August 22nd, 1918, this child was of normal weight (3.1 kg.), was breast-fed for six weeks, and then received diluted cow's milk, with additions of sugar, till six months old. Full milk and cereal were then given for a month. At seven months this was largely replaced by malt-soup (*Malzsuppe*), a preparation with calorie value equal to full milk, consisting of 33 per cent. milk, 4 per cent. flour, and 10 per cent. malt extract. The child thrived poorly from the start, and the weight curve deviated more and more from the normal until about the tenth month.

The total calories (approximate) and daily ration of milk fat, together with the energy quotient, are plotted on the chart underneath the weight curve. There is no evidence of lack of calories in the diet throughout this period; the energy quotient varied from 120 to 150 during the first six months, and later (seven to ten months) it varied from 150 to 180; but, nevertheless, the child only put on 400 grams weight in this period. It is evident, therefore, that the high calorie intake had little effect in stimulating growth. The inhibiting factor appears to have been the deficiency of antiscorbutic material in the diet. Severe symptoms of scurvy were noticed at eight and a half months (April 29th, 1919), after a period of about seven weeks in which malt-soup figured largely in the diet. As demonstrated by Hess and Fish (1914), malt-soup is especially poor in antiscorbutic properties, for the milk is diluted 1 in 3 with water,

and the food is boiled twice during the preparation. It is therefore possible that the malt-soup diet precipitated the onset of acute symptoms. The diet was changed at once to include a plentiful supply (1,000 to 600 c.cm.) of raw full milk, and growth began to take place slowly for the first month while the symptoms of scurvy remained, but later more rapidly. At 12 months the child weighed 5.5 kg., and at 16 months 8.1 kg. During this period there was no increase of calories or of fat, and the energy quotient progressively declined in value.

The prescurbutic period was marked by an attack of furunculosis. The scurvy attack was of a particularly severe character, the right thigh showed skin haemorrhages and was swollen and tender and the leg was maintained in a flexed position; blood cells were present in the urine. The acute symptoms yielded but slowly to treatment with raw milk, and after forty-one days (June 13th, 1919) haematuria still persisted. A persistent deformity (shortening of the right leg) was left; x-ray photographs taken in October, 1919, five months after the acute attack, when the child was 14 months old,

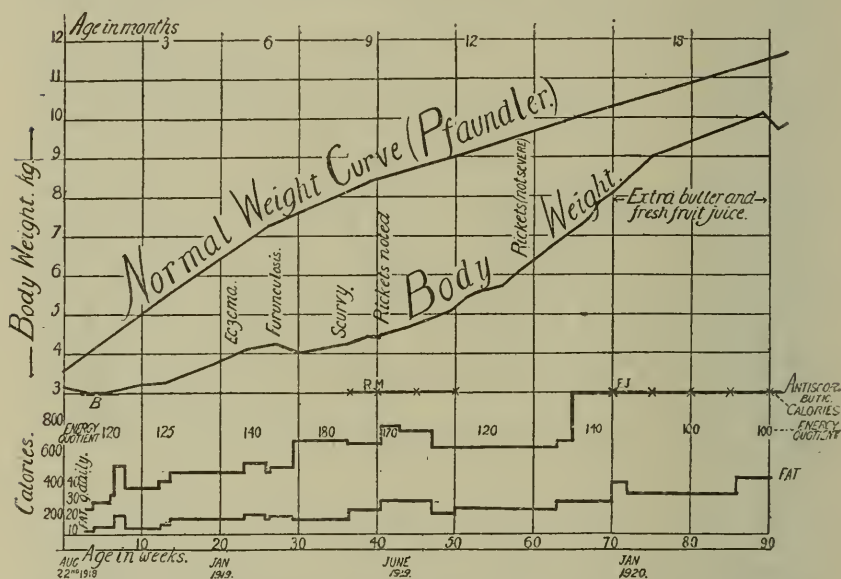


CHART 1.—Case 4, Barbara J., born August 22nd, 1918. B., Breast milk. R.M., Raw cow's milk. F.J., Raw fruit juice. E.Q., Energy quotient of diet.

showed structural deformity of the right femur, with 1 cm. shortening of the thigh as compared with the left; the left femur was also affected, but to a less degree. At this period a definite "rosary" and slightly bent tibiae are recorded as evidence of rickets.

Two months later, in December, 1919, the child was first seen by us. She was then 16 months old, and was receiving the mixed diet usual in the institution, consisting of milk, cocoa, puddings made with milk and cereals, finely sieved cooked vegetables, and a little bread. It was calculated that this diet had a calorie value approximately equal to 1,000 calories daily and contained 30 grams daily of milk fat. The weight curve was approaching the normal, but she was still 20 per cent. under weight for her age; her fontanelle was open; she had four teeth, but made no attempt to speak. The deformity of the right leg was still very obvious; she could sit up unsupported, but could not stand. She received a regular anti-scorbutic in the form of 20 grams daily of raw swede juice or orange juice. The milk fat in her diet was gradually increased

from about 30 grams daily to 35 to 40 grams, and later some cod-liver oil was given.

She made good progress during the succeeding five months, put on over 2 kg. in weight, the teeth erupted rapidly, the fontanelle was closed, there was marked increase in general intelligence and activity, and she learnt to walk.

This was the only child of the series who appeared to be unable to tolerate cod-liver oil, which caused loss of appetite accompanied by loss of weight, and consequently had to be discontinued.

Summary.

1. Marked failure to grow during the first nine months of life, associated with lack of antiscorbutic material in the diet, culminating in an attack of acute scurvy.

2. Growth in weight restored on inclusion of raw milk in the diet.

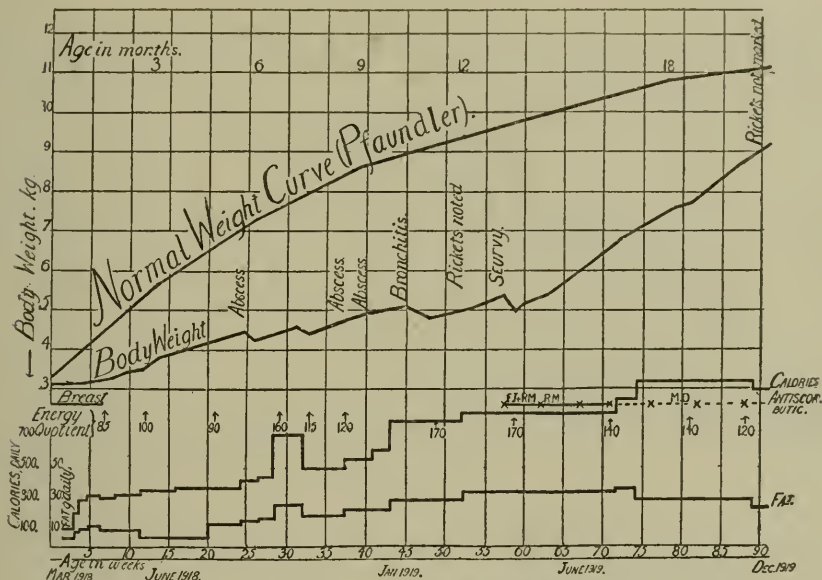


CHART 2.—Case 4A, Marie B., born March 18th, 1918. B., Breast milk. R.M., Raw milk. F.J., Fruit juice. M.D., Mixed diet.

3. Permanent deformity of scorbutic origin in long bone of the skeleton.

4. An attack of furunculosis during the prescorbutic period, possibly due to a diminished resistance to infection.

5. Good progress from 16 to 21 months, when extra milk fat and antiscorbutic were added to the diet.

The next case offers interesting confirmation of the conclusions drawn from Case 4 as to the effect upon growth of a deficiency of antiscorbutic material in the diet. This child left the home shortly after our first visit in December, 1919; she received no treatment from us, and her case is not therefore included in the accompanying table.

CASE 4A. (Chart 2.)

At birth, on March 18th, 1918, the child was of average weight (3 kg.); she received some breast milk for six weeks, after which the diet consisted of diluted cow's milk and sugar, and from the third to the fifth month included a considerable

amount of malt-soup. From the fifth to the twelfth month some full milk and cereal were given.

During this period growth in weight was very poor, and at 12 months the child's weight was about half that normal for the age. There had been no lack of calories in the diet (see Chart 2), but until the age of six months the proportion of milk fat was very low and never exceeded 20 grams daily. Later this improved, and from 10 to 12 months 30 grams of milk fat was taken daily. There was a deficiency of antiscorbutic material in the diet, especially when the malt-soup was given.

The prescorbutic period was again punctuated by various infections, furunculosis was noted at 6 months of age, again at 8 months and at 9 months; the child also had an attack of bronchitis at 10½ months.

At 12 months some degree of rickets was noted. At 13 months (April, 1919) acute symptoms of scurvy were recognized; blood was found in the urine, the gums were swollen, and the lower half of the right thigh was swollen and tender. The thorax was

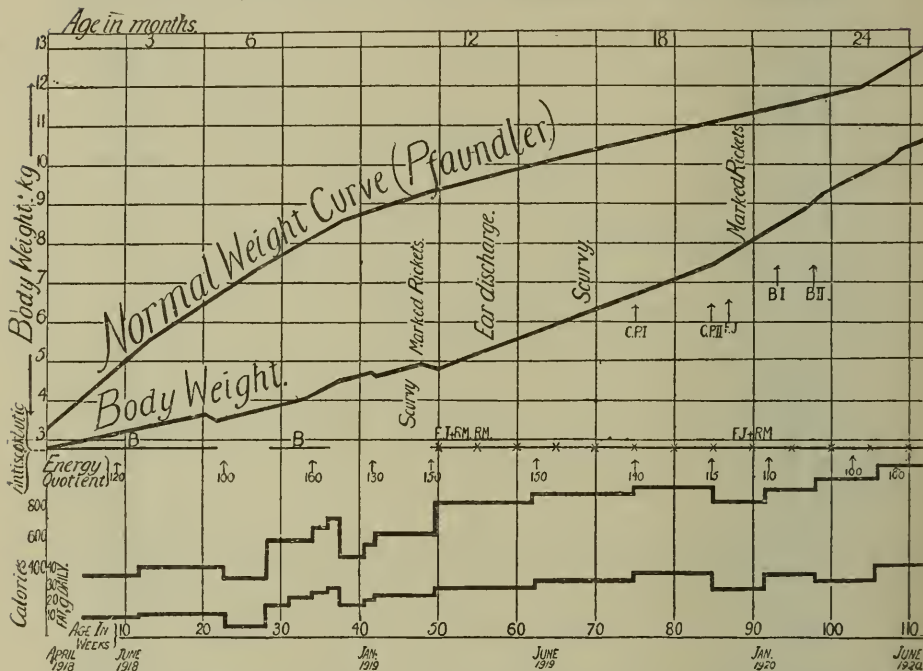


CHART 3.—Case 7, Friedrich B., born April 4th, 1918. B., Breast milk. R.M., Raw milk. F.J., Raw fruit juice. B. I, Butter 10 grams daily; B. II, butter 20 grams daily. C.P. I, 10 grams cod-liver oil containing phosphorus; C.P. II, cod-liver oil and phosphorus discontinued

flat and the epiphyses thickened but not sensitive. Fruit juice was given and raw milk; this treatment was continued for three months. Growth in weight took place immediately this antiscorbutic treatment was begun; the weight, which was 5.2 kg. at 13 months, improved to 6.8 kg. at 16 months, and 9.2 kg. at 21 months, and was fast approaching the normal (11.4 kg.). Mixed diet containing vegetables was introduced at 17 months.

When seen by us at this time she was making progress although backward: she could stand but did not walk, she had eleven teeth but made no attempt to speak.

CASE 7. (Chart 3.)

F. B. weighed 2.8 kg. at birth on April 4th, 1918. He also showed poor development during the first year of life. Breast-feeding was partial (300-350 c.cm. daily) until 5 months old, but at this age the weight had increased only to 3.6 kg. The

Case No.	Sex		Treatment (daily).	History of Scurvy and Other Illnesses.
1. J. U.	M	Severe in head and chest; enlarged intelligence; rachitic deformities less arrested, though deformities persist;	10 g. antiscorbutic. 10 g. cod-liver oil. Cod-liver oil 20 g. from April 15.	
2. J. R.	M); slight signs only of rickets; good only of rickets; 6 teeth; fontanelle of epiphyses the only sign of rickets;	20 g. antiscorbutic from Jan. 10, 1920. 400 g. extra milk from Feb. 10, 1920.	Scurvy at $8\frac{1}{2}$ months.
3. R. H.	M	n; good muscular power but activity 9 months; rachitic stigmata in rib eth. mata; fontanelle closed; 12 teeth. d epiphyses the only sign of rickets;	20 g. antiscorbutic. 10 g. cod-liver oil from Feb. 18, 1920.	Scurvy at $8\frac{1}{2}$ months.
4. B. J.	F	Spontaneous movements not vigorous; ward child, still suffering from effects 1 cm. shortening. in rickets; fontanelle almost closed; activity normal for age; dentition	20 g. antiscorbutic. 20 g. butter. 10 g. cod-liver oil in addition from April 15 to May 12, 1920.	Scurvy, $8\frac{1}{2}$ to 10 months; furunculosis 6 to 7 months.
5. W. W.	M	Spontaneous movements; pallid; no t cot; pallor less marked; epiphyses d; 7 teeth; intelligence and activity one'.	20 g. antiscorbutic. Butter, 10 g. from Jan. 1, 1920; 20 g. from Feb. 17, 1920. 10 g. cod-liver oil in addition from April 15, 1920.	Scurvy at 10 $\frac{1}{2}$ months; furunculosis.
6. A. D.	M	er good, but activity poor and mental ties of head, chest, and legs; tibiae; fontanelle almost closed; 8 teeth. ollowing severe attack of scurvy. mally intelligent. Rickets arrested. al for age; intelligent; deformities	20 g. antiscorbutic. Butter 10 g.; 20 g. from Feb. 17, 1920.	Scurvy, 7 to 11 months; furunculosis.
7. F. B.	M	ment; all tissues flaccid and muscular being raised; child lies inert and s much enlarged; fontanelle widely wer improved but legs still feeble; s marked; fontanelle closed; colour ion; rachitic stigmata retrogressing; ement in activity and intelligence; y.	20 g. antiscorbutic. Butter 10 g.; 20 g. from Feb. 20, 1920. 10 g. cod-liver oil from April 15, 1920.	Scurvy, 11 to 15 months.
8. I. P.	F	Issues soft and muscles very flaccid; neous movements; rickets shown in of tibiae; fontanelle open; 4 teeth. ings; marked pallor. ill pallid; fontanelle open; 4 teeth. scular power still feeble; fontanelle and tries to talk. 2 teeth; is intelligent and contented.erry, and plump; general condition aining in head and chest.	As for Case 7.	Two attacks of scurvy, at 8 months and 13 months
9. V. J.	F	Lead up only for a short time; body passively in cot and takes no notice fontanelle open; 13 teeth. arms and legs, and rests on elbows. proved. d intelligent; general development losed; 16 teeth.	As for Case 7.	Three attacks of scurvy, at 16, 20, and 24 months. Ear discharge at 9 months. Tuberculous finger 12 to 15 months.

Case No.	Sex.	Date of Birth, and Wt. in Kg.	Date of Examination.	Age (Mths.)	Duration of Treatment (Mths.)	Body Weight (Kg.)	Normal for Age (Kg.)	Difference (Kg.)	Description.	Treatment (daily).	History of Scurvy and Other Illnesses.
1. J. U.	M.	7.12.18	22.11.19	12	0	6.2	9.4	3.2	Cannot sit. No attempt to use legs; rickets severe in head and chest; enlarged epiphyses; no teeth.	10 g. antiscorbutic.	
		(2.0)	5.3.20	14	2½	8.2	10.1	1.9	Moves actively; stands in cot; shows signs of intelligence; rachitic deformities less obvious; no teeth.	10 g. cod-liver oil.	
			14.5.20	17	5	9.6	10.8	1.2	Can stand unsupported; tries to talk; rickets arrested, though deformities persist; tissues firm; muscular power good.	Cod-liver oil 20 g. from April 15.	
2. J. R.	M.	5.1.19	22.12.19	12	0	5.9	9.4	3.5	Can sit; tries to use legs; fontanelle open (2 cm.); slight signs only of rickets; good muscular power; two teeth.	20 g. antiscorbutic from Jan. 10, 1920.	Scurvy at 1½ months.
		(3.5)	13.4.20	15	3	8.9	10.	1.4	Stands well with support; colour good; traces only of rickets; 6 teeth; fontanelle still open.	400 g. extra milk from Feb. 10, 1920.	
			16.6.20	13	5	9.2	10.8	1.4	Fontanelle closed and firm; slight enlargements of epiphyses the only sign of rickets; can stand alone and walk round cot; 6 teeth.		
3. R. H.	M.	10.10.18	22.12.19	14	0	8.2	9.9	1.7	Can sit, but makes no attempt to stand; well grown; good muscular power but activity poor; general development as for child of 9 months; rachitic stigmata in rib junctions and epiphyses; fontanelle open; 4 teeth.	20 g. antiscorbutic. 10 g. cod-liver oil from Feb. 18, 1920.	Scurvy at 8½ months.
		(2.9)	13.4.20	18	4	9.9	10.8	0.9	Active; stands in cot; no progress in rachitic stigmata; fontanelle closed; 12 teeth.		
			15.6.20	20	6	10.8	11.7	0.9	Can just walk; tone of all tissues good; enlarged epiphyses the only sign of rickets; development normal for age.		
4. B. J.	F.	22.8.18	10.12.19	16	0	8.1	10.3	2.2	Can sit but cannot stand; tone of tissues good; spontaneous movements not vigorous; fontanelle open; 4 teeth; rickets slight. Backward child, still suffering from effects of a severe attack of scurvy; right femur shows 1 cm. shortening.	20 g. antiscorbutic. 20 g. butter. 10 g. cod-liver oil in addition from April 15 to May 12, 1920.	Scurvy, 8½ to 10 months; furunculosis 6 to 7 months.
		(3.1)	5.2.20	17½	1½	9.1	10.7	1.6	Stands and can walk with assistance; no progress in rickets; fontanelle almost closed; marked general improvement.		
			14.5.20	21	5	9.9	11.4	1.5	Condition good; child vigorous and intelligent; activity normal for age; dentition proceeding rapidly; rickets stationary.		
5. W. W.	M.	5.8.18	5.12.19	16	0	8.7	10.3	1.6	Can sit but cannot stand; well grown; makes no spontaneous movements; pallid; no obvious sign of rickets; 5 teeth.	20 g. antiscorbutic. Butter, 10 g. from Jan. 1, 1920; 20 g. from Feb. 17, 1920.	Scurvy at 10½ months; furunculosis.
		(2.9)	5.2.20	18	2	9.7	10.8	1.1	Stands with support and moves vigorously about cot; pallor less marked; epiphyses slightly enlarged.	10 g. cod-liver oil in addition from April 15, 1920.	
			8.5.20	21	5	11.6	11.45	—	Can stand alone; condition good; fontanelle closed; 7 teeth; intelligence and activity normal for age (three weeks later could walk alone).		
6. A. D.	M.	30.6.18	22.1.2.19	18	0	9.5	10.8	1.3	Child sits and can stand up in cot; muscular power good, but activity poor and mental condition lethargic; marked rachitic deformities of head, chest, and legs; tibiae bowed; shortening and disproportion of limb; fontanelle almost closed; 8 teeth. Child had fracture of the radius at 12 months following severe attack of scurvy.	20 g. antiscorbutic. Butter 10 g.; 20 g. from Feb. 17, 1920.	Scurvy, 7 to 11 months; furunculosis.
		(4.1)	3.2.20	19	1½	10.7	11.1	0.4	Stands well; tries to walk and to talk; seems normally intelligent. Rickets arrested.		
			13.4.20	21½	4	12.0	11.6	—	Excellent condition of all tissues; activity normal for age; intelligent; deformities less marked; 10 teeth.		
7. F. B.	M.	4.4.18	23.12.19	21	0	8.1	11.4	3.3	Very backward; no attempt at spontaneous movement; all tissues flaccid and muscular power very defective; limbs fall limply after being raised; child lies inert and resents handling; head rachitic and epiphyses much enlarged; fontanelle widely open; 10 teeth.	20 g. antiscorbutic. Butter 10 g.; 20 g. from Feb. 20, 1920.	Scurvy. 11 to 15 months.
		(2.8)	4.2.20	22	1½	8.7	11.7	3.0	Can sit alone and can kneel in cot; muscular power improved but legs still feeble; 15 teeth; is making rapid progress.	10 g. cod-liver oil from April 15, 1920.	
			5.3.20	23	2½	9.4	11.9	2.5	Can stand; rickets arrested and deformities less marked; fontanelle closed; colour improved.		
			16.6.20	26	6	10.9	12.3	1.4	Child beginning to walk; all tissues in good condition; rachitic stigmata retrogressing; head contour improved; remarkable improvement in activity and intelligence; marked disproportion of limbs to length of body.		
8. I. P.	F.	11.2.18	23.12.19	22	0	7.6	11.6	4.0	Very backward; cannot sit even with support; tissues soft and muscles very flaccid; child passive and makes no attempt at spontaneous movements; rickets shown in moderate degree in head, chest, and lower ends of tibiae; fontanelle open; 4 teeth. Child shows no intelligent interest in surroundings; marked pallor.	As for Case 7.	Two attacks of scurvy, at 8 months and 13 months.
		(1.6)	4.2.20	24	1½	8.3	12.1	3.8	Tries to sit; is more active; rickets stationary; still pallid; fontanelle open; 4 teeth.		
			3.3.20	25	2½	8.9	12.2	3.3	Can sit alone, but makes no attempt to stand; muscular power still feeble; fontanelle closed; 6 teeth. Child appears more intelligent and tries to talk.		
			13.4.20	26	4	9.8	12.3	2.5	Marked improvement in activity; tries to crawl; 12 teeth; is intelligent and contented. Child can stand and walk round cot; is active, merry, and plump; general condition excellent; slight signs of rachitic stigmata remaining in head and chest.		
9. V. J.	F.	12.5.17	5.12.19	31	0	8.1	12.7	4.6	Very backward; cannot sit alone and can hold head up only for a short time; body limp and helpless; child pallid and feeble; lies passively in cot and takes no notice of being handled; slight signs only of rickets; fontanelle open; 13 teeth.	As for Case 7.	Three attacks of scurvy, at 16, 20, and 24 months. Ear discharge at 9 months. Tuberculous finger 12 to 15 months.
		(2.8)	3.3.20	34	3	9.5	13.0	3.7	Begins to sit up; activity much improved; uses arms and legs, and rests on elbows. Interest shown in her surroundings; colour improved.		
			14.5.20	36	5	10.8	13.1	2.3	Can stand up in cot; tissues firm; child active and intelligent; general development as for a child of 18 months; fontanelle almost closed; 16 teeth.		
			1.6.20	37	5½	10.9	13.3	2.4	Beginning to walk; traces only of rickets.		

artificial food given had consisted of malt-soup, and after breast-feeding was discontinued malt-soup formed the entire diet for a short period. At 6½ months the weight was only 3.8 kg.; breast milk was again introduced, supplemented by increasing amounts of full cow's milk. At 8½ months the weight was 4.4 kg., and the diet was changed to diluted cow's milk and cereal food.

At 11 months (weight = 4.9 kg.) definite symptoms of scurvy, swollen gums, and red blood cells in the urine were noted. Lemon juice and some raw milk were given; the scurvy symptoms improved slowly and were still apparent after four months' treatment, but the effect of antiscorbutic material in the diet was evident in the weight curve, which showed a slow but distinct improvement. The raw milk was continued for ten months (December, 1919), when the child was 21 months old and weighed 8 kg.

At this period we first saw the child in the backward condition summarized in the accompanying table. He could hold up his head but could not sit unsupported. His muscles were flaccid, the body fell limply forward, and if arm or leg were handled it would fall back helplessly when released. There was no spontaneous movement, muscular power was that shown by a normal baby of three months; the child resented handling and cried continuously. Dentition was not markedly backward and there were ten teeth. The contour of the head was very rachitic, the fontanelle was widely open and the epiphyses at wrists and ankles were enlarged. After this examination the anti-scorbutic treatment of 150 c.cm. raw milk daily was increased (December, 1919) by the addition of 20 c.cm. of potent material in the form of raw turnip juice or orange juice. Butter was given (10-20 grams daily) and later 10 grams of cod-liver oil in addition, so that the daily fat ration reached 35-45 grams.

The following is a short summary of his progress :

Three weeks (January 16th, 1920). Weight 8,460 grams. Some improvement in muscular power, can sit up with some support but still feeble, and makes no attempt to use legs. More contented and sleeps better.

Six weeks (February 4th, 1920). Weight 8,710 grams. More active, can sit alone, and kneel in cot holding on to the bars with hands.

Ten weeks (March 5th, 1920). Weight 9,390 grams. Stood for first time; rickets less prominent in head contour, tibiae slightly bowed, chest contour good.

Twenty weeks (May 14th, 1920). Weight, 10,630 grams. Fine looking child, active, skin healthy, subcutaneous tissues firm, stands easily. Rickets much less apparent, head contour much improved, no parietal, and only slight frontal, bossing, tibiae straight, chest contour normal; wrist epiphyses show slight swellings.

Twenty-four weeks (June 16th). Weight 10,900 grams. Beginning to walk.

The improvement in weight is well seen in Chart 3, the 30 per cent. deficiency from the normal at 21 months is reduced to a 10 per cent. deficiency after five to six months' treatment.

Summary.

1. This case shows a period of poor growth in weight, culminating in an attack of acute scurvy; improvement in growth in weight on adding antiscorbutic material to the diet.

2. Marked improvement in growth and also general progress and activity on adding a larger ration of antiscorbutic, and at the same time increasing the milk-fat.

CASE 8. (Chart 4.)

Ida P., born February 11th, 1918, was one of twins, and was much under normal weight at birth (1.6 kg.); her twin brother weighed 1.4 kg. She received a complete diet of breast milk for nearly three months; then followed a transitional period of mixed feeding for a few weeks, after which the diet was completely artificial, and consisted of diluted milk and cereal food. This was continued for four months; at six months of

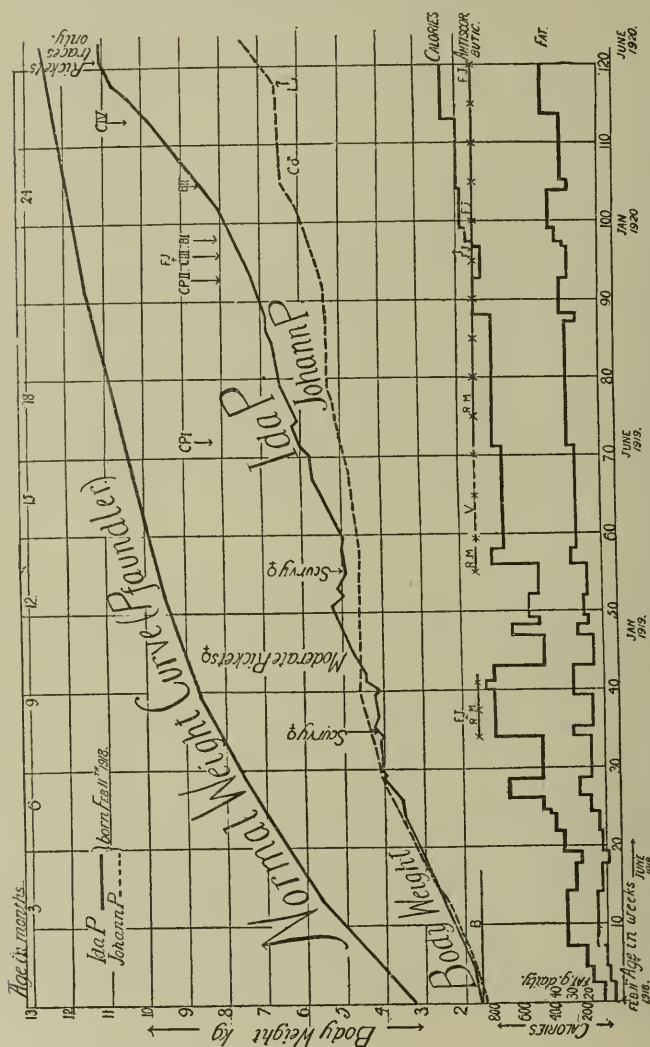


CHART 4.—Case 8, Ida P., born February 11th, 1918. B., Breast milk. R.M., Raw milk. V., Vegetable food. F.J., Raw fruit juice. C.P. I, Cod-liver oil with phosphorus 5 grams daily; C.P. II, cod-liver oil and phosphorus discontinued; C. III, cod-liver oil 5 grams daily; C. IV, cod-liver oil 10 grams daily. B. I, Butter 10 grams daily; B. II, butter 20 grams daily. Dotted curve = Johann P., her twin brother. C. δ , Cod-liver oil given. L.J., Raw lemon juice. Curves representing daily calorie and fat intake and antiscorbutic in diet refer to Ida P.

age (September, 1918) definite symptoms of scurvy were observed. The child screamed on being handled, swellings were noticed on the left thigh and left shoulder, the epiphysis of the left humerus was swollen and painful, the muscles over the right tibia were swollen and oedematous, and skin haemorrhages were noted on chest, neck, and back. Raw full milk was introduced into the diet (800 to 600 c.cm.), fruit juice was given and an infusion of pine tree needles. The symptoms cleared up slowly, and after two months raw milk was discontinued and scurvy was considered to be cured. At this time (ten months) a moderate degree of rickets was noted.

During the first four months of life on breast milk the child made fair progress and the weight curve ran fairly parallel to the normal. There was marked flattening during the period preceding the scurvy attack and while the symptoms lasted. The weight, which had reached 4 kg. at 6½ months, remained stationary until 9 months, when the child weighed only 4.2 kg. After the definite symptoms of scurvy had disappeared some progress in weight was noted; although some full milk was retained in the dietary it was not given raw, and both fat content and antiscorbutic value were low. The short period of growth from 9 to 12 months was succeeded by another stationary period from 12 months (5.3 kg.) to 15 months (5.5 kg.). During this period there is a continuous record of coughs, feverish colds,



Ida P. (left) and Johann P. (right), June 22nd, 1920, at 28 months of age.

etc., and in March, 1919, at 13 months of age, a second attack of acute scurvy took place, shown by a swollen, painful femur and presence of blood in the urine. Treatment with whole raw milk was again instituted, and at 14 months vegetables were also introduced into the diet. Growth in weight began slowly and proceeded evenly until December, 1919, when we first saw the child. Raw milk in decreasing amount had been continued with slight intermission till this date.

The child was then 22 months old, and weighed 7.6 kg., about 35 per cent. below normal. She was limp and passive, and her muscles were flaccid; she could neither sit nor stand, nor was there any attempt at spontaneous movement. She showed a moderate degree of rickets; she had four teeth, the fontanelle was still open, and distinct frontal bossing was present. The chest was rachitic in contour, with projecting sternum, palpable

rosary, and a lateral sulcus at the rib junctions. The legs were straight, but there was a slight thickening of the lower ends of both tibiae. The wrists were not enlarged.

During the six months she was under observation she received daily 20 grams antiscorbutic material (raw lemon juice, swede-turnip juice, or orange juice) and 10 grams of butter. In February, 1920, the butter was increased to 20 grams, and in April 10 grams of cod-liver oil were given in addition. There was a dramatic improvement and the weight curve began to make a steep ascent towards the normal; five and a half months of treatment (up to June, 1920) reduced the deficiency in weight from 35 per cent. to 11 per cent. The child was then $27\frac{1}{2}$ months old and weighed 11.1 kg. (normal for that age 12.5 kg.).

The rapid increase in weight during this period was accompanied by marked general progress, which is summarized in the Table. After five to six months of the enriched diet the little girl (at 28 months) was a transformed creature; she was active, merry, and plump, could stand and walk round her cot, and was trying to talk. She had twelve teeth, her fontanelle had closed, and there was a very striking remission in the signs of rickets noted on our first examination. No rosary and no swellings of the epiphyses were to be felt, and the chest contour had much improved. (See photograph.)

An unexpected control for this case was discovered in a twin brother, who had also been in the institution since birth but remained in another ward in the same building. The salient points of his past history were obtained, and his weight curve is plotted alongside that of his sister's (see dotted weight curve in Chart 4), and runs fairly parallel with it.

The brother suffered also from a severe attack of scurvy during the first year of life, and in his case the symptoms appear to have been present persistently from the ninth to the eighteenth month. During this period the body weight increased only from 4.2 to 5.3 kg., so that at 18 months of age the child was about half the normal weight. After this period various additions to diet were given in form of cod-liver oil and meat juice, but no regular antiscorbutic was given, and the weight curve remained very unsatisfactory until May, 1920, when he received for the first time raw lemon juice. This change coincided with a sharp upward turn in his weight curve.

It was at the end of this period that we first saw the child, who was feeble, anaemic, and unable to sit up without support. His arms and legs were thin and ill developed, his condition was lethargic, and he showed no signs of intelligence. The contour of the head was rachitic, the fontanelle was closed, but there was marked frontal and parietal bossing, the chest was compressed laterally, there was a definite rosary, and the abdomen was prominent. His condition exactly reflected that of his twin sister six months previously before her diet was enriched. The contrast between the two children is well shown in the accompanying photograph.

We are inclined to attribute the improvement in the twin sister principally to the effect of a regular and potent antiscorbutic in the diet, for the proportion of fat, though increased also, was not so greatly altered, and

the child also had been taking a small amount of cod-liver oil daily before she came under our notice.

CASE 9. (Chart 5.)

V. J., aged $2\frac{1}{2}$ years old when first seen, was the oldest of the series and at the same time the most backward. She was about 35 per cent. under weight for her age, did not even hold up her head, and made no attempt at spontaneous movement.

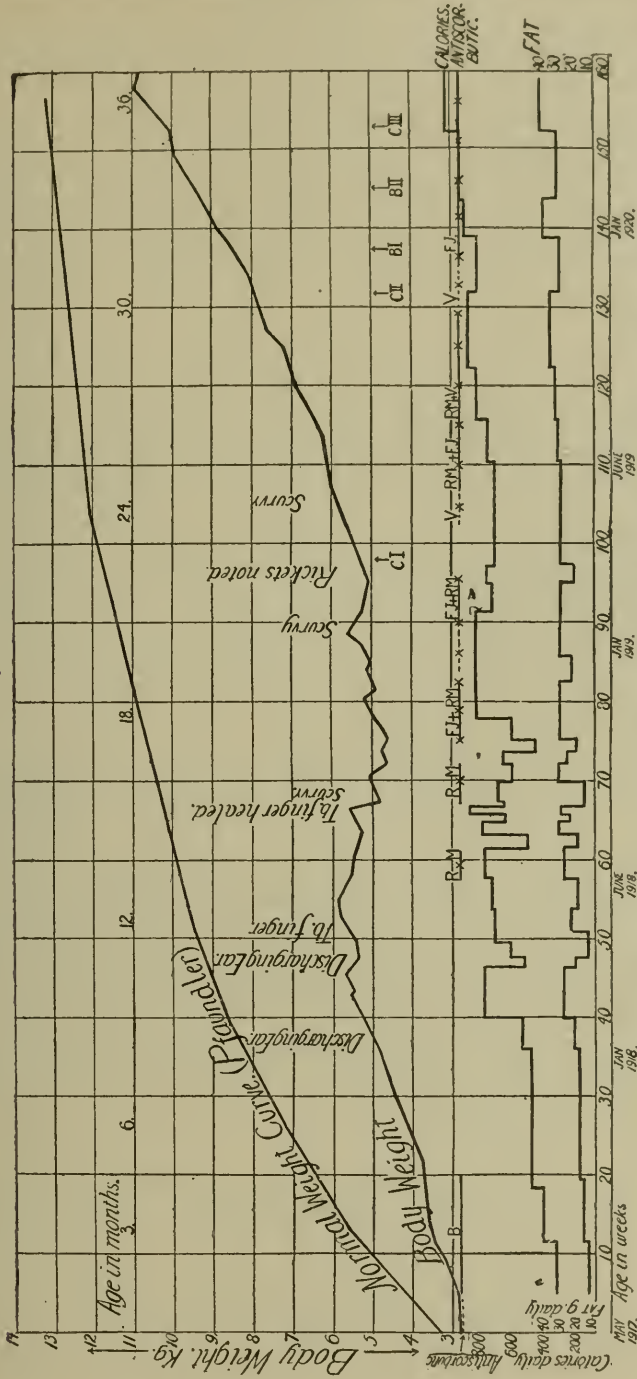


CHART 5.—Case 9, Victoria J., born May 12th, 1917. B., Breast milk. R.M., Raw milk. V., Vegetable food. F.J., Fruit juice (raw). C. I, Cod-liver oil with phosphorus given; C. II, cod-liver oil and phosphorus discontinued. B. I, Butter given 10 grams daily; B. II, butter increased to 20 grams daily. C. III, Cod-liver oil given in addition, 10 grams daily.

She weighed 2.8 kg. at birth (May 12th, 1917), and after one month of breast-feeding received additions of diluted milk. From 4½ months old her diet was entirely artificial, and until the age of 9 months consisted of milk diluted two-thirds with sugar and cereal. Small amounts of full milk were also given. From 9 to 11 months of age (February and March, 1918) she received full milk and cereal food made with full milk. During the first year of life the weight curve became progressively flatter and departed more and more from the normal.

The second year of life (May, 1918, to May, 1919) was even less satisfactory than the first. The net increase in weight over the whole twelve months was negligible, and amounted to 200 grams, and the year was marked by three attacks of scurvy.

The diet for the first month of this period contained a large proportion of "malt-soup," but in June, 1918, there is a record of full raw milk given for a few weeks, indicating that a scorbutic condition was suspected, although no symptoms are noted in her dossier. The diet then reverted again to ordinary milk and cereal food, until, at the end of August, 1918 (at 13½ months) malt-soup again made its appearance in the diet, with the addition of 100 grams daily of raw milk. In spite of this anti-scorbutic addition definite scurvy was diagnosed in September (at 16 months). Fruit juice was given, and the diet was again changed to raw full milk, which was continued, with a short interval, until December, 1918. A cooked diet was then given of whole milk and cereal. Vegetables were included, but in January, 1919 (at 20 months), definite scurvy symptoms were again noted in the form of swollen gums and swollen and painful legs. Raw apple juice and lemon juice and raw milk were given again, and the raw milk was continued until the beginning of March (1919). The acute symptoms had then disappeared, so the raw milk was again discontinued and replaced by malt-soup. At the beginning of May (the child was then 24 months old) definite scurvy was diagnosed for the third time, and on this occasion was more severe in character than on the two previous occasions. There were skin haemorrhages on the back and thigh, the thigh was sensitive and painful, the gums were swollen, there was oedema of both feet, and haematuria.

Note was made of rickets at 22 months, the child had only five teeth, and was evidently very backward.

The curative treatment of this third attack of scurvy consisted of lemon juice, green vegetables, and raw full milk, which on this occasion formed a large proportion of the total diet, and was continued for nearly six months. The child began to grow, and at 2½ years had increased from 5.9 to 7.9 kg. in weight. At this period the child was seen for the first time, and her condition is summarized in the table. She was 2½ years old, and, although very backward in general progress, showed no gross signs of rickets. The head was well ossified and properly developed, the fontanelle was wide but not soft, the chest was rather contracted, but the abdomen was flat. The legs were long and straight and the body well proportioned, the arms were very weak, and the wrists showed enlargement. The muscular power was extremely feeble, and the head could only be held up for a few minutes. The child was lethargic, unintelligent, and inactive.

The effect of a combination of antiscorbutic and fat-soluble accessory factors is well shown in the progress made when raw lemon and orange juice 10 to 20 grams, and 10 to 20 grams of butter were added to the diet (see Table). Later 20 grams daily of swede-turnip juice was substituted for orange juice. In six weeks the child was trying to sit up; in three months she could do so, and was trying to stand; she also showed activity and intelli-

gence in many ways. In five months she was beginning to walk with help.

This case is in many ways the most instructive of the series. First, it shows very clearly the inhibition of growth and progress imposed by a scorbutic or pre-scorbutic condition, even when definite symptoms were absent. In the second place, the history of the second year of this child's life shows that scurvy must not be considered as cured when the severe symptoms are no longer obvious. The curative treatment for the first two attacks was applied for a short period and discontinued when symptoms had disappeared; on both occasions a speedy relapse took place. Only after the third attack of scurvy was the antiscorbutic treatment continued for a prolonged period, when the child showed by its growth that the constitutional disturbance was being really rectified.

CONCLUSIONS.

It is not possible to make generalizations upon the history of nine children, but the following provisional conclusions are stated here in the hope of suggesting further research in this field:

1. The addition of antiscorbutic juices and of fats containing the fat-soluble accessory factor was found to have a satisfactory result in stimulating growth and progress of nine very backward children, of ages varying from 12 to 31 months. The condition of these children before treatment showed that development had been retarded for many months.

2. The diet allotted for the children did not appear to be lacking in calories, but there was evidence that their food had been deficient in antiscorbutic principle and in milk fat.

3. It appears that deficiency of antiscorbutic material in diet had been an important cause of failure to grow. Eight of the nine children gave a history of previous attacks of definite scurvy, and two (Cases 4 and 6) showed bony deformities which were probably of scorbutic origin.

4. It is not possible to determine whether the beneficial effect observed after enriching the diet with two accessory factors is to be attributed to the extra antiscorbutic or to the extra fat given; probably both factors were concerned. In some instances—for example, Cases 4A and 8—improvement in growth and remission of rachitic symptoms were observed after addition of antiscorbutic material to a diet already containing a fair allowance of milk fat.

5. The cases studied indicate that the child's capacity for recovery is considerable when conditions of deprivation are rectified; the normal standard could be approached in six to twelve months, even after twenty-four months of retardation in growth and progress.

In conclusion, our warm thanks are due to Dr. G. Riether, Director of the Landes Zentral Kinderheim, for hospitality afforded to us in the wards of the institution and for permission to publish these observations. We also wish to express our indebtedness to Primarius Dr. M. Zarfl, physician-in-charge of the section in which these observations were made, for his continued co-operation and sympathy with us in our work.

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No 3

HUNGER - OSTEOMALACIA IN VIENNA, 1920. ITS RELATION TO DIET.

BY

ELSIE J. DALYELL, M.B., CH.M. SYDNEY,

BEIT MEMORIAL FELLOW ;

AND

HARRIETTE CHICK, D.Sc.,

LISTER INSTITUTE, LONDON.

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HUNGER-OSTEOMALACIA IN VIENNA, 1920.

ITS RELATION TO DIET.*

Introductory.

TOWARDS the end of 1918 a condition with symptoms comparable to those of the earlier stages of osteomalacia was prevalent in Vienna. X ray photographs of some of the cases showed osteoporosis, and in a few instances spontaneous fracture occurred. The disease was described by Edelmann (1919), Schlesinger (1919, I. and II.), and Schiff (1919). Schlesinger pointed out that this disease to which he gave the name "hunger-osteomalacia" differed from osteomalacia associated with pregnancy in its slower development and more chronic character. Further, the pelvis was seldom affected, marked deformity was uncommon, and the majority of patients were elderly individuals of either sex. The disease occurred chiefly among the poorest section of the population, but many cases were noted also among middle-class people in reduced circumstances. All observers expressed the opinion that it was in some way connected with the food deprivation then existing in Vienna. In the late summer of 1919 there was a marked remission in the number of cases (Schiff, loc. cit.). Our investigations began in the autumn of the year 1919, and inquiries were first made in October and November, regarding the occurrence of the disease, but no cases were found. In December, however, fresh and recurrent cases were seen in two convents in which the nuns had suffered severely during the previous winter, and simultaneously there appeared in the hospitals, both as out-patients and in-patients, persons suffering from the disease in

* The clinical observations contained in this paper were made by Dr. Dalyell.

various degrees of severity. From this date until the late spring of 1920 there was no lack of material for study.

By arrangement with Prof. Dr. Arthur Schiff, investigations were undertaken in his out-patient department at the Central Verband der Krankassen, one of the principal medical centres of the Health Insurance Organisation in Vienna. At this centre medical examination took place of all persons applying for sick pay through inability to work. Between January, 1920, and May, 1920, over 600 patients diagnosed as cases of hunger-osteomalacia attended for medical advice; of these 204 were selected for special study. These patients attended regularly for examination and special treatment, and observations were made of their progress by Miss M. Hume in collaboration with Dr. E. Nirenstein. A detailed account of this investigation is published in a separate paper.

In addition to the ambulatory cases in convents and at the Krankenkassa, special observations were made on 18 cases admitted to the III. Medizinisches Klinik of the Allgemeines Krankenhaus by permission of Prof. Dr. H. Schlesinger, and upon eight cases in the I. Med. Klinik of the University (Prof. K. F. Wenckebach), in collaboration with Dozent Dr. Porges.

Onset, Symptoms, and Course of the Disease.

The short description of hunger-osteomalacia symptoms given by Schlesinger and Edelmann has reference to severe cases admitted to hospital as in-patients. In these the painful areas were commonly the chest and the sacral region, the ribs were invariably sensitive to pressure, and less often pain was felt in the extremities. Bones of the face were affected in about one-third of the cases, the pelvis was rarely painful, and pelvic deformity was not observed. There was great pain on movement, and in cases not completely bedridden there was a characteristic stiff and waddling gait, and the climbing of stairs was specially difficult. Frequently the patients declared that they had decreased in height. Edelmann described the condition of his patients as prematurely aged.

The variety of material that was available allowed our observations on the disease to be made at all stages of its course.

The initial symptom was difficulty in walking, owing to pain in the tarsal and metatarsal bones, generally accompanied by œdema of the feet and ankles. The œdema when

present subsided after some weeks, but without diminution of pain on walking. The lumbar and sacral regions were next affected, and stooping or walking was accompanied by severe pain in these areas, of an intense and persistent nature. Thoracic symptoms developed later with pain on compression of the ribs, progressing from lower to upper ribs; pain was often felt also on coughing or deep breathing. Arms, shoulders, and hands were not so often affected, and face symptoms were present only in the most severe cases.

The disease was afebrile, and progressed from the early condition with mere discomfort in walking to a bedridden state in which any movement of the body caused agonising pain. The following description applies to the ambulatory cases of moderate severity which came most commonly under observation. The patient had a characteristic expression, the face sallow, the eyes sunken and with an expression of suffering. The skin was dry, harsh, and loose, the body anæmic, flabby, and ill-nourished, but not usually emaciated. Movement was painful, and in walking the body was bent forward and, as a rule, support of a stick in one or both hands was necessary. The waddling gait and the difficulty in mounting steps were so characteristic that it was easy to detect sufferers from the disease among the people in the street, especially when endeavouring to enter trams or vehicles. The legs were widely spaced, the feet hardly lifted from the ground in short shuffling steps, and the body moved stiffly from side to side in the endeavour to limit as much as possible swinging movement at the hips. Attempts to step upwards, as in mounting stairs, failed unless a support could be grasped with the hands and the body pulled up by the shoulder muscles. The pains were always associated with movement. As long as the patient could remain motionless, whether lying, sitting, or standing, there was little discomfort; but movements in which the trunk muscles were involved, as in bending, turning, sitting up, or walking, caused acute pain which was always worse with the preliminary muscular effort.

The hands, arms, and shoulders, being seldom affected, were much used in carrying out movements. Thus the patient would change from lying to sitting, or from sitting to standing, with the help of arms and hands; and in a severe case would get in or out of bed only after flexing the legs by lifting the thighs with the hands over the edge of the bed. The defective action of the body muscles was not due to loss of power, but to voluntary limitation of movement owing to apprehension of pain. Many movements only became painful when pressure or weight was exerted on the parts concerned. For example, if the patient could support the body by grasping a fixed object with the hands, the foot could be lifted from the floor and the leg flexed at the hip and knee without obvious discomfort, but a similar movement to carry the body up a step was often impossible, from the severe pain produced by throwing the weight of the body on one side of the pelvis.

Systematic examination of patients showed certain areas where manual pressure was almost invariably painful. Such pressure points were always over bones. The joints were rarely involved and muscular masses were never sensitive. The tarsal bones, especially the head of the astragalus and

the proximal ends of the metatarsals, were usually sensitive, also the lower ends of the tibia and fibula, and the anterior and inner borders of the tibia in its lower half; the lower two-thirds of the shaft of the femur was occasionally sensitive. The knee-joint was rarely involved, and the hip-joint was always free from pain. The most sensitive pressure area was the sacral and lumbar region—e.g., over the sacro-iliac joints and transverse processes of the lumbar vertebrae. Severe pain was always felt in this area during stooping or bending movements, and this symptom was always marked and persistent. The iliac crests, pubic rami, and pubic symphysis were seldom affected, and deep pressure through the abdominal wall on the inner surface of the ilium was never painful. Bony deformity of the pelvis did not occur in any case seen by us.

Sudden abduction of the flexed thighs usually caused severe pain over the lesser trochanter and at the site of the insertion of the adductor muscles, a symptom attributed to sudden tension of the ilio-psoas and adductor muscles. This formed one of the chief diagnostic features of the disease and was an important indication of its progress or remission.

In almost every case, sudden compression of the ribs caused acute pain, felt most severely in the axillary line. The lower four ribs were commonly affected; in severer cases the fourth-eighth ribs also, and the upper ribs were rarely involved. Here also the pain was evidently associated with tension of the intercostal muscles. The thoracic vertebrae and posterior rib junctions were not sensitive nor were the costochondral junctions and costal cartilages. Pressure on the sternum sometimes caused pain but it was not often severe. The bones of the face were sensitive only in certain advanced cases and this symptom yielded readily to treatment. When present the painful pressure points were over the supra-orbital ridges, malar bones, nasal bones, and along the mandible.

The muscles were not painful when handled, showed neither wasting nor other deformity, and their contractile force was not diminished. It was therefore unlikely that the whole muscle was involved in the existing lesion. The nerve reflexes were normal, and there was no evidence of peripheral neuritis nor of damage to nerve trunks, so that it was improbable that the symptoms of the disease could be due to nerve involvement only. The association of tetany with the disease was not infrequent. This is a point of special interest and is dealt with in a later section.

The degree to which bones were involved in the lesion was difficult to determine. The sensitive areas were limited. Bony deformity was not seen by us, but has been described by Eisler (1919), Eisler and Hass (1921), and Schlesinger. In their observations the affected areas included ribs, pelvis, spinal column, and femur. Evidence of a pathological change in the substance of the bones was obtained by us from a few patients who gave definite history of decrease in the height of the body (cf. Schlesinger, 1919, I., and Case 4 in Appendix). In one instance also spontaneous fracture of the femur occurred in a woman aged 32. Spontaneous fractures were observed by Edelmann also (loc. cit.).

Although it was not possible to say with certainty how much of the bone substance was affected, it was evident that the areas of acute pain on pressure corresponded to the points of attachment of certain muscle groups—e.g., lumbar muscles, adductor muscles of the thigh, and the ilio-psoas. There was no opportunity for examining any histological specimens which might throw light on the process which took place at the painful sites, as we have no record of a fatal termination of any case of the disease that came under our observation. As far as we are aware the disease progressed until the patient was completely immobilised in bed, and succumbed ultimately to intercurrent disease (usually pulmonary) or to starvation, which unhappily was not an impossibility in Vienna in 1918–20.

Occurrence of Tetany.

Occurrence of tetany with osteomalacia has frequently been noticed and is of much interest. In 22 cases of hunger-osteomalacia Schlesinger (1919, II.) observed marked tetany in two cases (elderly women of 62 and 55 years respectively), and extra excitability of the muscles in 15 cases. He calls attention to the facts (1) that tetany is otherwise a rare condition in elderly people, and (2) that in cases of "hunger-osteomalacia" accompanied by tetany, symptoms of the latter disappeared simultaneously with those of osteomalacia. In two instances a subsequent relapse was again accompanied by tetany. Schlesinger (1920) distinguishes this condition as "hunger-tetanie," and points out that the symptoms are often asymmetrical in their distribution. Sauer (1920) described the case of a woman, aged 29 years, suffering from marked tetany and simultaneously from severe hunger-osteomalacia.

Definite tetany was observed by us four times in a total of 39 cases, but many patients complained of some degree of cramp in the feet, legs, or forearms. The most marked case that came under our notice occurred in a woman of 52, suffering from severe hunger-osteomalacia (see Case 5 in Appendix), who showed altered electrical response of the muscles, Erb's sign, Trousseau's sign, and Schlesinger's sign (tonic spasm of the foot when the leg is flexed at the hip to a right angle). In every case seen by us the symptoms of tetany subsided gradually after the patient was admitted to hospital. Contrary to Schlesinger's observation that tetany and osteomalacia symptoms were relieved simultaneously, in the case

mentioned above (Appendix, Case 5), the tetany manifestations were greatly relieved before the treatment had shown much effect upon the osteomalacia symptoms.

Age- and Sex-Incidence.

Hunger-osteomalacia may be regarded as a disease which affects specially the portion of the population above middle age. Hume and Nirenstein's (1921) analysis of 131 cases treated as out-patients at the Zentral Verband der Krankenkassen shows that 115 (or 88 per cent.) were between the ages of 40 and 75 and 16 (or 12 per cent.) were under 40 years of age. Our notes of 43 cases show 36 (or 84 per cent.) between 40 and 70 years of age, and 7 (or 16 per cent.) between 20 and 40. Of 19 cases of hunger-osteomalacia studied by Edelmann (*loc. cit.*) in 1918 to 1919, 15 were women and 4 were men. In the cases observed by us in 1920 there was also a much higher proportion of women than men, but these included cases in convents. In the general population in 1920 the numbers of men and women affected were more nearly equal. Of 177 cases in which sex was noted by Hume and Nirenstein 99 (or 56 per cent.) were women and 78 (or 44 per cent.) were men. In this outbreak of hunger-osteomalacia, as compared with the better known osteomalacia of pregnancy, the most striking features were the incidence among elderly and old people, and the extent to which men were affected.

Influence of Occupation.

Evidence was not obtained to show that hard manual labour was a special contributory cause of hunger-osteomalacia. If the condition results from food deprivation, analogy with other deficiency diseases suggests that a relation would exist between the incidence of the disease and increased physical strain. Age, however, had more influence than occupation in determining the incidence of hunger-osteomalacia, and the hardest manual work was performed by individuals of an age (20-40) when some degree of protection from the condition seems to be afforded naturally. Moreover, in the existing economic crisis artisans and manual labourers were better paid and could secure a more abundant supply of food than other sections of the population, so that this additional advantage helped to protect such workers. Poverty and old age predisposed to the disease, and in many cases the food deprivation was specially severe and prolonged. In the convents the dietary was rigidly reduced, due partly to poverty

and partly to the isolation in which these communities were placed. The lack of food was so extreme that younger occupants of the convents suffered as severely from the disease as the older nuns, and sisters appointed for light teaching duty were as much affected as those responsible for gardening and heavy household tasks.

Seasonal Incidence.

The seasonal incidence of hunger-osteomalacia was interesting. Many recurrent cases gave a history of first appearance of symptoms in winter 1918 or early spring 1919, with remissions in summer 1919 and relapse in the winter of 1919-20. The fresh cases seen by us had begun to suffer in the cold weather of the late autumn of 1919. The relief experienced in summer weather was probably due to higher temperature, but it is also possible that the disease was alleviated as a result of the improved diet associated with the summer season.

Ætiology.

The literature is full of observations, connecting osteomalacia and other disorders affecting the calcification of the bones with the activities of the ductless glands. A comprehensive abstract of the papers upon this subject is given by Zuntz (1912). Important observations in this field are those of Erdheim (1906) who, after extirpation by cautery of the parathyroids, produced a condition of tetany in rats, as well as abnormal dentine formation. Later (1914) the same observer found abnormal histological conditions in the parathyroids in a series of rats which developed rickets spontaneously. In addition (1907) he found at autopsy pathological changes in the parathyroids of human subjects who had suffered from osteomalacia of pregnancy. The theory advanced by Erdheim is that the normal secretion from the healthy parathyroid may have neutralising effect upon that of other glands, such as the ovary, which may, in certain conditions, affect the normal course of bone formation. Some support for this view is found in the association of osteomalacia with multiple pregnancy (Sonntag) and in the beneficial effect of castration upon such cases as described by Fehling (1891; 1895). Other observers have recorded the good effect of treating osteomalacia with adrenalin (Bossi (1907), Edelman. loc. cit.); and extract of the hypophysis was employed with good effect in osteomalacia by Bab (1911), Neu (1911), Pal (1912), and in rickets by Klotz (1912). Biedl (1916) reached

the opinion that the cause of osteomalacia is to be found in the disordered action of more than one gland.

It has been well recognised also that external conditions have an influence upon the incidence of osteomalacia. Sonntag (1905) states that although everywhere a rare disease it is unusually frequent in certain places, such as the valley of the Rhine and its tributaries, Lombardy, the Danube Island Schütt in Hungary, and the Harz Mountains. The disease is comparatively rare in North Germany, in the United States, and in England, where it is described only in association with pregnancy. Sonntag also connects the occurrence of the disease in pregnancy with damp dwellings, poor diet, insufficient clothing, hard work, and excessive care and worry, in a word with poverty, and he calls attention to the good effect which improved conditions of life exert upon the disorder. It is the opinion of Schlesinger that more careful observation and accurate diagnosis will discover the disease to be more widely distributed than has been hitherto supposed.

The causation of osteomalacia has hitherto been generally ascribed to disordered calcium metabolism, attributable to abnormal action of endocrine glands and associated with poverty and destitution. This conception of its ætiology is also adopted by Edelmann and Schlesinger, who consider that hunger-osteomalacia is brought about by the effect of under-nourishment upon the activities of one or more of the ductless glands. The fact must be emphasised, however, that the connexion between "hunger-osteomalacia" and osteomalacia that occurs in pregnancy is far from clear. Despite the widespread distribution of "hunger-osteomalacia" among the older section of the population of Vienna, no increased incidence of the typical osteomalacia of pregnancy was reported among child-bearing women, and the conditions favourable to the occurrence of "hunger-osteomalacia" must, in general, be regarded as different from those that determine the onset of the disease in pregnancy.

Specific Deficiencies in Diet as a Possible Cause of Hunger-Osteomalacia.

The consideration of diet as a factor of specific importance in producing osteomalacia of pregnancy does not seem to have been undertaken. Fehling, however, refers to the possibility of averting the disease by improved diet. In the outbreak here

recorded the question of diet was of primary importance, and prophylaxis and cure were effected by diet alone.

In considering external conditions in Vienna during the past five years, the most striking unusual circumstance has been the food deprivation suffered by the population. There is no doubt in the minds of those who studied the outbreak of hunger-osteomalacia in Vienna in 1918-19 that the condition was, indirectly at least, dietetic in origin. This is made clear by the resolution introduced by Prof. Dr. Schiff, and adopted unanimously by the Gesellschaft der Aerzte at their session on March 14th, 1919, calling the attention of the Inter-Allied Food Mission to this famine disease, and begging for quick and generous supplies of food as the best means of dealing with it.

The food supplies of the city of Vienna diminished progressively from 1917 onwards, and during the winter of 1918-19, and of 1919-20, were reduced to a minimum. The diets of the patients examined by us had consisted mainly of bread and vegetables, with small amounts of flour and sugar. Milk, butter, and eggs were unknown; meat was not consumed, the price being prohibitive, and the only fat in the diet was a small and irregular supply of lard. It was the opinion of some that the disease, although confined at that time to the poorest class in the city, was not strictly a starvation disease, but was rather the result of a qualitative deficiency in the diet caused by lack of certain definite foods. The patients were always thin, but not emaciated, and while the process of cure in hospital was invariably accompanied by increase in body-weight, the successful treatment of out-patients was accomplished without any such gain in weight. Lack of fat was the most striking deficiency in the patients' diets prior to the development of osteomalacia.

The value of certain fats in diet has been much emphasised recently by the results of experimental work upon growth in young animals.

The work of Falta and Nogerrath (1905), Stepp (1911, 1912), of McCollum and his co-workers (1913-1916), and of Osborne and Mendel (1913, 1915), has shown that in young rats growth may be entirely inhibited by a diet adequate in calories and in protein supply if certain fats are absent, and will be restored if a small amount of these fats be again included in the diet. This result was attributed by McCollum not to the fat in itself but to the presence of a certain vitamin or accessory factor dissolved in the fat; this vitamin he called the "fat-soluble A factor." It is present to some extent in most fats, but those of animal origin, such as fish-liver oils, beef fat, mutton fat and butter, contain it in greater concentration, cod-liver oil being particularly rich

in this vitamin. The existence of this factor in green leaves has also been established.

It is to be remembered that one view of the ætiology of the analogous disease rickets is that the disease may be attributed to a lack of fat in the diet of infants (Cheadle (1906), Still (1907), Holt (1907)). In Austria cod-liver oil containing an addition of phosphorus is a classic remedy for rickets, and has been widely used for osteomalacia, but the view commonly held is that the beneficial result that follows is to be ascribed to the phosphorus rather than to the oil, although the investigations of Schabad (1909, 1910) upon calcium metabolism in rickets, before and during treatment, have shown that the cod-liver oil rather than the phosphorus is to be regarded as the active constituent in increasing the retention of calcium. Schabad also investigated the effect upon calcium metabolism of sesame oil and, in conjunction with Sorochowitz, of olive oil. These oils were found to influence calcium retention in a degree which was insignificant by comparison with that of cod-liver oil.

These results of Schabad have received interesting confirmation from the recent work of Mellanby (1918, 1919) upon experimental rickets in young dogs. Rickets occurred in puppies fed upon diets poor in fat-soluble vitamin—i.e., containing fat in the form of olive oil or lard, but not when cod-liver oil or butter was substituted for these fats. Mellanby has advanced the view that rickets is a dietetic disease caused by a deficiency in the diet, not of fat per se, but of some factor contained in certain fats. Three studies on experimental rickets in rats have appeared recently by McCollum, Simmons, Parsons, Shipley and Park (1921). In their experiments rachitis and similar disturbances of osteogenesis were produced by deficient diets, and they have demonstrated that some substance in cod-liver oil causes calcium to be deposited in the same fashion in which deposition occurs in spontaneous healing of rachitis. Their observations lend support to the view that an anti-rachitic factor present in animal fats plays some rôle in the normal process of ossification. Hess (1917, 1920, I. and II.), while admitting the prophylactic or curative action of cod-liver oil upon rickets in children, considers that lack of the fat-soluble A vitamin is only one among several factors concerned in causation of the disease.

Concurrently with the outbreak of "hunger-osteomalacia" in Vienna, during 1919 and 1920, there has been an unusual prevalence of rickets among infants and young children, and of late rickets among young adults. This suggests that all these disorders may have a common origin, and may represent a reaction to some unusual circumstance interfering with normal metabolism and the proper nutrition of the bony tissues.

The identity of rickets and real osteomalacia from the anatomical standpoint has been strongly supported by the histological work of Pommer (1885), Looser (1908, 1920), and others. The different theories which have been advanced regarding the ætiology of osteomalacia and of rickets form two

parallel series. For each disease there are theories involving (1) disordered action of the ductless glands, (2) defective hygiene, (3) defective diet, and (4) bacterial infection (Morpurgo, 1900, 1902, 1907). The occurrence of tetany with osteomalacia, and the association of this condition in childhood with rickets, form another important parallel between the two diseases. With such indications of correspondence between rickets and osteomalacia, it was of the greatest interest to ascertain whether evidence in support of a similar dietetic theory for both diseases could be obtained during the outbreak of hunger-osteomalacia in Vienna.

Result of Dietetic Treatment upon "Hunger-Osteomalacia."

From earlier observations it was known that simple rest in bed with good diet was a successful form of treatment for the lighter cases of hunger-osteomalacia. Cod-liver oil containing phosphorus was highly esteemed as a curative substance, the phosphorus being regarded as the active agent. When cod-liver oil could no longer be obtained in Austria, different vegetable oils, with additions of phosphorus, were employed for treatment. In certain cases good results were reported from the use of phosphorus in the form of pills without oil (Schlesinger, 1919, I.); this treatment, however, was not tried with out-patients, but coincided with admission to hospital and a generally improved dietary. A similar criticism applies to the good results from the use of extract of thyroid gland reported by Edelman (loc. cit.).

In view of the possible connexion between the known high content of cod-liver oil in fat-soluble A accessory factor, and the efficacy of the oil in the treatment of osteomalacia, it was desirable to try to determine the therapeutic value, in this disease, of various fats known to contain the fat-soluble A accessory factor in different degree. The results obtained are set out in detail below. They show that the symptoms could be relieved, in absence of any other treatment, by one each of the series of fats employed, but that cod-liver oil was the most potent. Zilva and Miura (1921) have succeeded in making quantitative estimations of the relative content of A vitamin in different fats, and in experiments with rats have found some samples of cod-liver oil to be 250 times as potent as butter.

TABLE I.—21 Cases of Osteomalacia in Convents.

The additions to the diet were arranged to be approximately equal in caloric value.

Group.	Addition to diet.	Approx. amount daily, g.	Calories.	Fat per day, g.	No. of cases.	Result.
(a)	Sugar or syrup. Cereal. Olive oil.	15 40 15	330	15	10	Good progress in 5 slight cases; no progress in 5†.
(b)	Margarine.* Olive oil.	25 20	380	43	5	Good progress slightly inferior to (c).
(c)	Butter. Cod-liver oil. Egg (dried).	25 10 = 1 egg	360	40	11†	Good progress.

* This sample of margarine was believed to be manufactured from vegetable fats, it has since been ascertained that it contained 80 per cent. animal fats.

† Later including 5 from group (a).

‡ Three of the 5 cases in which no progress was made were severe, and showed good improvement when treatment (c) was substituted.

TABLE II.—18 Severe Cases of Osteomalacia.

Observations taken in the III. Medizinische Abteilung at the Allgemeines Krankenhaus (Prof. Dr. H. Schlesinger), February–April, 1920. The effect of various additions to the ordinary hospital diet.

(a)	Sugar or syrup. Rice or other cereal.	60 100	550	0	5	See below (1).
(b)	Margarine.* Olive oil.	50 (25 at first.) 15	540	60	} 13	Steady progress in 8 cases; very slow progress in 5.
(c)	Butter. Egg (dried).	50 (25 at first.) = 1 egg	400	52		
(d)	Cod-liver oil.	60	540	60	4	See below (2).

(1) Very slow improvement in 20–30 days; marked progress when changed to (b), (c), (d).

(2) Marked improvement in 4 severe cases, making slow progress on (a), (b), and (c).

The food supplies of Vienna in general were most irregular, and it was not possible to arrange special diets which could be controlled in every particular. Except for a few important cases we had to be content with watching the effects of various additions

to the ordinary diet, whether in hospital, convent, or home. Without exception such ordinary diets were always deficient in fat and always scanty. The following foods were, therefore, chosen for the different groups into which the patients were divided, and in comparative observations the additions made were roughly equivalent in calorie value. (a) Sugar and cereals; (b) vegetable oils (margarine, olive oil, &c.); (c) butter and eggs; (d) cod-liver oil; (e) vegetable oil containing phosphorus; (f) green vegetables. After admission to hospital some improvement usually followed from rest in bed, and relief from the misery caused by moving about, but such effects were only temporary, and relapse occurred if no increase were made in the fat-content of the dietary. (Tables I. and II.)

(a) *Influence of Sugar and Cereals—i.e., extra calories without extra fat.*—These additions were made to the ordinary hospital diet of five severe cases in the Allgemeines Krankenhaus (see Table II.). Slow improvement was noted in three to four weeks, but only one case progressed sufficiently to leave hospital; the other four cases required a further period with treatment by addition of (b), (c), or (d) before they could be discharged (e.g., Case 2 in the Appendix). Ten cases in a convent (see Table I.) also had their diets enriched with extra cereals and sugar, but here a small amount of olive oil (15 c.cm. daily) was also added. Progress was noted in five of the lighter cases only, and not in the remaining patients. (Table I., group (a).)

(b) *Influence of Vegetable Fats and (c) Butter and Eggs.*—The treatment of a series of cases with these additions was arranged (see Tables I. and II.) in the belief that the ordinary vegetable oils of commerce might be regarded as free of the fat-soluble accessory factor, a view in accord with results obtained by McCollum and by Osborne and Mendel and their co-workers (*loc. cit.*). Such additions would therefore have demonstrated the effect of adding fat to the diet uncomplicated by the presence of the vitamin. We found, however, that the effect of vegetable fats differed only in degree from that of butter and cod-liver oil, since cures followed the use of each of the fats administered, but when vegetable oils were used, the good effect appeared more slowly and in severe cases was less complete. (See Case 1 in Appendix.) The margarine employed was selected in the belief that it contained only vegetable oil, but on subsequent inquiry from the manufacturers it proved to contain 80 per cent. of animal fats. No marked difference was observed in the response to treatment of 24 cases divided into two groups, one of which received margarine and olive oil, and the other butter and egg as dietetic additions. (See Tables I. and II., groups (b) and (c).)

(d) *Cod-liver Oil.*—This was the most effective fat tried, and no case failed to recover when this oil was added to the dietary in sufficient amount. Doses of cod-liver oil equal to 60 g. daily effected speedy cure in severe refractory cases

after other forms of treatment had failed. Cases 5, 6, 7 in the Appendix are instructive instances.

Case 6. George R., aged 60, was specially severe, suffering acute pain with the least movement. As additions to the hospital diet, he received margarine and olive oil in amounts equal to 65 g. of fat daily for 40 days, but no definite change took place in his condition. 30 g. of cod-liver oil daily were then given for seven days, and were not effective; but on increasing the daily dose to 60 g. cod-liver oil there was a dramatic improvement in his symptoms within a week, and in three weeks his gait was easy and painless. This case illustrates well the necessity for giving the requisite amount of the right sort of fat.

Case 7. Christina B., was the most severe case encountered. She had been unable to walk for three months before entering hospital, could not stand without support, and was helpless except for slight movements in bed made with the help of her hands. In addition to the ordinary hospital diet she received at first 25 g. and later 50 g. of butter and one egg each day for 31 days, and for part of this time phosphorus pills were also given. At the end of this period scarcely any improvement was noticeable. After changing her extra allowance to 60 g. of cod-liver oil daily, marked progress was evident after five days and the patient was discharged after 14 days of this treatment.

One very refractory case was that of Mater U. (Case 4, Appendix), a delicate woman, aged 52, who could not tolerate cod-liver oil. Treatment for 17 days with 20 g. butter daily, plus extra milk and eggs, was very disappointing, but after increasing the butter ration to 50 g. daily and the milk to $\frac{1}{4}$ litre, there was slow steady improvement and the patient was able to walk without her stick after 10 days. Another severe case in the same convent (Mater P., aged 57, Appendix, Case 3) treated at the same period with 20 g. butter daily and with 15 g. cod-liver oil instead of milk or eggs, made rapid progress and admitted immediate relief from pain.

(e) *Influence of Vegetable Oil to which Phosphorus was added.*—Miss Hume and Dr. Nirenstein made a special series of investigations to determine the comparative value of cod-liver oil and of vegetable oil containing phosphorus with 131 out-patient cases selected from about 500 at the central station of the Verband der Krankenkassen. A detailed analysis of these observations is published by the authors in a separate communication, but they have permitted their general conclusions to be stated here. Cod-liver oil was found to be definitely superior to rape oil containing phosphorus (0.01 per cent.), and large doses of the plant oil (200 g. weekly) gave a rate of improvement about equal to that obtained with smaller doses of cod-liver oil (100 to 150 g. weekly).

(f) *Influence of Green Vegetables.*—In order to throw light on the question whether fat or the fat-soluble accessory factor was to be regarded as the effective agent in treatment, it was important to ascertain whether green vegetables (another source of the fat-soluble A vitamin) had any curative effect on this disease. The history of relief in summer and return of symptoms in winter given repeatedly by recurrent

cases suggested that this might be the case. It was difficult to put the theory to a practical test, for most of the patients were old or elderly and feeble, and a diet devoid of fat and containing large amounts of cabbage, salads, and spinach would not have been tolerated. Also it was impossible to arrange such a diet in the period January–April, 1920, when most of our observations were made. An attempt was made, however, in the case of a woman, aged 30, who entered hospital at the beginning of May, suffering severely from hunger-osteomalacia. Her diet was strictly controlled and contained as much green vegetables as could be tolerated and 5 g. only of fat in the form of olive oil. The patient consumed an average of 700 g. of green vegetables daily, but made no appreciable progress in 18 days. Her diet was then changed and the green vegetables replaced by 30 to 40 g. of cod-liver oil and 20 g. butter daily, and improvement took place rapidly after the change. The case is described in detail in the Appendix, Case 8.

General Character of the Recovery.

The general character of the recovery was always the same, but occurred more rapidly in hospital patients than in those treated as out-patients and carrying on their ordinary occupations at home. After a few days on effective treatment body movements became less painful and the patient could stoop or turn more readily. The gait often improved within a week, and although it retained a waddling character it was less stiff and showed a more natural movement at hips and ankles. Within the second week there was often great remission in the pressure pains, and the abduction pain was usually less. As a rule the pains caused by compression of the ribs continued longer, and the upper ribs were free from pain earlier than the lower ribs. The most obstinate symptoms were always the pains on movement in the lumbo-sacral region and in the tarsal bones, but these disappeared within 4–6 weeks, and did not tend to recur if suitable amounts of fat were taken regularly as a prophylactic.

The eight cases described in detail in the Appendix were selected to illustrate various types of symptoms and different methods of treatment. In many the comparative value of the respective additions to the diet is well seen from the alteration in the rate of recovery when one treatment was substituted for another.

Summary and Conclusion.

1. The occurrence of "hunger-osteomalacia" in Vienna during the period of greatest food deprivation, its incidence among the poorest inhabitants and the beneficial effect of improved diet without any

other form of treatment, suggested that the disease was of dietetic origin.

2. The disorder affected chiefly middle-aged and old people of both sexes. The characteristic symptoms of the condition are pain on body movement, a waddling gait, difficulty in mounting stairs, severe pain in sacral region on pressure or movement, and pain in the ribs on compression of the thorax. Tetany may occur in association with the disease.

3. Forty-eight cases were treated by making additions to patients' diets in the form of carbohydrates, cereals, and fats of various kinds.

4. Little improvement could be demonstrated after addition to the diet of sugar or cereals (i.e., extra calories without fat), although in many instances the previous diet had been very scanty.

5. Recovery followed the addition of either cod-liver oil, butter, oleo-margarine containing 80 per cent. of animal fat, or olive oil. The beneficial influence was in the order named above, cod-liver oil being far the most effective. Some of the severer cases did not improve until cod-liver oil was given.

6. That the relative therapeutic value of the fats used corresponds roughly with their content in the fat-soluble A vitamin is regarded as significant.

7. It is also possible to interpret the remission of symptoms of "hunger-osteomalacia" in summer and their relapse in winter as due to varying supplies of vitamin A in the diets, for, during summer, inclusion of green vegetables in the diet provides a valuable source of this vitamin. One attempt, however, to cure a severe case with a fat-free diet, rich in green vegetables, was not successful. More observations are needed on this point.

8. The increase in Vienna of rickets in children and late rickets in young adults, simultaneously with the occurrence of "hunger-osteomalacia," suggests that the three disorders may be due to the same cause.

In conclusion we wish to express our thanks to those members of the medical profession in Vienna who have afforded us the opportunity of carrying out this investigation. We are specially indebted to Prof. H. Schlesinger, of the III. Medizinische Abteilung of the Allgemeines Krankenhaus, who permitted us to make special study of cases in his wards, and gave us the benefit of his previous extensive experience. Our thanks are also due to Prof. K. F. Wenckebach and Dozent Dr. Porges of the I. Medizinische Klinik of the University, and to Prof. Dr. Schiff of the Zentral Verband der Krankenkassen; also to those members of the nursing staff who

cheerfully undertook all the extra work involved in the preparation of special diets.

For the supply and transport of special foodstuffs we have to thank the British Section of the Inter-Allied Food Mission then stationed in Vienna; and we desire also to record our thanks to the Mission of the Society of Friends working in Vienna for much help and coöperation, especially in supplying from their stores particular articles of diet essential for our investigation.

APPENDIX.

Following are the notes on eight cases :—

CASE 1.—Antonia B., aged 48; admitted March 20th, 1920. Severe recurrent case: symptoms first noted in July, 1919, improved in August and September, 1919, and reappeared in January, 1920. On admission patient had shuffling gait and complained of great pain on walking and on all movements involving the trunk muscles. She had acute pain on compression of ribs and on sudden abduction of flexed thighs. Pain was elicited on pressure over inner border of tibia, shaft and condyles of femur, and over iliac crests; also along shaft of each humerus, especially at insertion of deltoid muscle.

Treatment.—60 g. of olive oil daily with carefully controlled diet of fat-free milk, cereals, pulses, and fruits. Calorie value of diet 2500 daily. Protein content 60–65 g.; fat content 60 g. This diet was begun on third day after admission and continued unchanged for 32 days, and the patient's weight increased by 3·3 kg. Progress—Gait improved within six days of admission. After nine days' treatment: distinct general improvement; gait still stiff but no longer waddling and patient can take longer steps; can get in and out of bed more easily; less pain on compression of ribs. Eighteen days: great progress; gait normal; can spring from bed without use of hands; no pain on abduction of thighs; pressure pain persist over lower ribs and sacrum, and over ankle-joints and tibiæ. Twenty-five days: pains in ankles still present; no other symptoms; discharged cured April 24th, 1920, but symptoms recurred.

CASE 2.—Anna H., aged 65; admitted Jan. 15th, 1920. Symptoms: stiff waddling painful movements on walking; acute pain on compression of ribs and abduction of thighs; some pain on pressure over pubic symphysis and rami.

Treatment.—(a) Diet enriched by abundant extra cereals and carbohydrates without addition of fat on eighth day after admission. After 15 days' treatment, all symptoms unchanged. (b) 50 g. margarine (containing 80 per cent. of animal fats) and 15 g. olive oil added to daily diet. After 10 days' treatment: gait much improved; pressure pains in ribs persisting. Seventeen days: normal gait; slight pain on compression of ribs; no other symptoms; patient discharged Feb. 26th, 1920.

CASE 3.—Mater P., aged 57. Treatment begun Jan. 10th, 1920, in convent. Symptoms present for past two years, with remissions during summer months. Severe case, and walks only with great difficulty. Marked pressure pain over

ribs, lumbar vertebræ, and sacro-iliac joints ; has pre-tibial œdema and when trying to walk has cramps in both feet.

Treatment.—(a) Extra calories, consisting of cereals and carbohydrates with 15 g. daily of olive oil. After 16 days' treatment, no improvement. (b) Butter 25 g., cod-liver oil 15 g., and one egg given as additions to the daily diet. After eight days' treatment, improvement in pressure pains and in body movements ; walking somewhat less painful. Twenty-three days : can walk rapidly without use of stick ; movements of body free and painless ; no pains on pressure over ribs or sacrum ; some pain and œdema persist in feet. General condition greatly improved ; prophylactic doses of cod-liver oil continued.

CASE 4.—Mater U., aged 52 ; treated from Jan. 10th, 1920, in convent. Symptoms have been present for two years and are specially severe. Patient emaciated, and gives definite history of decrease in height and shortening of arms. Walking and all body movements cause intense suffering, and pressure pains are acute over ribs, sacro-iliac joints, lumbar vertebræ, hip-joints, dorsum of foot, and calf muscles. Has tendency to spasm of foot.

Treatment.—(a) Extra calories as for Case 3. After 14 days' treatment, no improvement. (b) Butter 25 g. and one egg daily, in addition to ordinary diet ; cod-liver oil 15 g. daily was also ordered, but could not be tolerated by this patient and was not taken. After 17 days' treatment, no improvement. (c) Butter 50 g., one egg, and 250 c.cm. milk daily in addition to the ordinary diet ; total extra fat 60 g. After 14 days' treatment : marked improvement ; slight pressure pains persist over sacrum and lower ribs, but not elsewhere ; gait still stiff, but walking is no longer painful and for four days patient has not used her stick ; body movements in bending, sitting, or standing still limited but greatly improved. Prophylactic treatment continued with smaller doses of butter and milk daily.

CASE 5.—Maria B., aged 62 ; admitted Jan. 13th, 1920. A severe and refractory case, complicated by tetany. Symptoms have been present for past year in ribs and sacral region. For some months patient has been unable to walk owing to pain caused by body movement and has remained in bed ; she can turn in bed and raise herself only with great difficulty and with use of hands. Tetany well marked ; Trousseau's sign and Erb's sign positive.

Treatment.—(a) Extra calories in form of cereals and carbohydrates ; anti-neuritic vitamin also given as marmite, 10 g. daily. After 10 days' treatment : no change in general condition ; all symptoms still present, and tetany well marked. (b) Butter 25 g., one egg and marmite 10 g. daily in addition to ordinary hospital diet. After 10 days' treatment : no improvement ; patient is unable to walk, and pressure pains are unchanged ; tetany symptoms are less marked but still present. Since admission increase in body-weight of 2.4 kg. (c) Cod-liver oil 60 g. daily. After 10 days' treatment : marked progress ; can walk a little and gets in and out of bed without difficulty ; great diminution in pain on compression of ribs and on abduction of thighs ; knees still painful and slight ankle-clonus present ; tetany symptoms much less marked.

Twenty-one days : pressure pains persist though slighter in degree ; abduction test negative ; gait still stiff. Thirty-five days : pressure pains have disappeared ; no abnormality of gait, and patient walks slowly without pain : complains only of pain in ankle-joint and along shaft of femur on right side. Discharged March 15th, 1920.

CASE 6.—George R., aged 60 ; admitted Jan. 14th, 1920. Body-weight 58·5 kg., height 171 cm. Very severe case, with history of onset of symptoms nine months previously. First noticed cedema of legs and pains in ankles ; thighs and ribs were later affected. All movements are so painful that patient is now practically helpless ; cannot stand without support, and in bed can change position only by use of hands and arms. Has acute pain on compression of ribs and sternum, and on abduction of flexed thighs.

Treatment.—(a) Extra margarine 25–50 g., olive oil 15 g. daily, in addition to hospital diet. After 25 days' treatment : margarine 25 g. daily, no improvement. Margarine then increased to 50 g. daily for a period of 10 days ; slight improvement, but still unable to walk. (b) Cod-liver oil 30 g. daily, later 60 g. After two days' treatment : immediate diminution in pressure pains ; rib symptoms less marked ; walking no easier. Seven days : rib symptoms almost disappeared ; still cannot stand without support ; cod-liver oil then increased to 60 g. daily. Fourteen days : pressure pains and abduction pain very slight ; can walk slowly without support and with little pain. Twenty-one days : condition very satisfactory ; walks well and makes free movements of bending, sitting, and standing. Twenty-eight days : traces only of pressure pains elicited ; gait normal and patient walks without pain or fatigue. The general condition is very good and weight has increased 9 kg. since admission to hospital. Discharged March 22nd, 1920.

CASE 7.—Christina B., aged 50 ; admitted Feb. 5th, 1920. The most severe case that came under our notice. Symptoms have been present without remission since previous spring, and for four months before admission patient has been helpless in bed. Pressure pains over ribs, sternum, pelvis, and lumbo-sacral region are intense, and acute pain follows abduction of flexed thighs. Patient stands feebly when supported, but cannot take a step nor carry out any body movement owing to the agonising pain that accompanies muscular effort.

Treatment.—(a) Butter 50 g. and one egg daily, in addition to ordinary hospital diet. After 14 days' treatment : patient is freer from pain, can sit up in bed slowly and get in and out of bed with difficulty ; cannot walk. From this date phosphorus pills given, equivalent to 0·0005 g. phosphorus daily. Twenty-five days : no progress in movements and condition still very helpless ; pressure pains still acute, especially in ribs ; no result observed from treatment with phosphorus. (b) Cod-liver oil 60 g. daily to replace former additions to diet ; phosphorus pills omitted. After five days' treatment : pressure pains much less ; abduction test causes slight pain. Patient shows great improvement and has lost expression of suffering ; can sit up in bed without using hands, but still has great difficulty in getting

in and out of bed and has to flex thighs with hands ; can stand alone with feet far apart, and can take a few steps with support. Fourteen days : trace of pain on compression of ribs but not elsewhere ; can move from bed slowly without pain, stands without support, walks with help of stick ; has shuffling gait. Discharged March 17th, 1920.

CASE 8.—Rosa T., aged 30 ; bookbinder ; admitted May 20th, 1920. The unusual feature of this case was the occurrence of the disease in a severe form in so young a woman ; symptoms had been present and progressing for more than a year, but patient had only given up work two weeks before entering hospital. On admission gait was stiff and steps were short and shuffling ; all body movements, such as bending or getting into or out of bed, were difficult and painful and were only possible when aided by hands ; pain was caused by pressure over frontal, malar, and nasal bones, ribs, lumbar vertebræ, sacro-iliac joints, shaft of femur, and borders of tibiæ ; pain on abduction of flexed thighs very acute.

Treatment.—(a) Fat-free diet, with addition of green vegetables. Daily food-supply was equivalent to 2000 calories, and consisted of bread, rice, oatmeal, peas, beans, lentils, cocoa, sugar, and chocolate. In addition a large amount of green vegetables was consumed, the daily allowance being at first 400–500 g., and later 700 g. Vegetables used were spinach, lettuce, cabbage, cress, asparagus, and cucumber. For cooking purposes 5 g. of olive oil daily was allotted ; this diet was continued for 18 days. Progress : After 18 days' treatment patient had gained 1 kg. in weight. Distribution and degree of pains on pressure were unaltered ; gait was still stiff and body movements difficult though less so than before. Progress of case was not satisfactory and patient was disinclined to take larger amounts of green vegetables, so it was decided to discontinue treatment on these lines. (b) 60 g. of animal fat added to the diet daily, and no green vegetables given ; cod-liver oil 30 g., butter 20 g., and one egg were given daily and 50 g. of dried full milk were substituted for the fat-free milk. Total calories 2500 ; protein 60 g. After 12 days' treatment : gait much more free, and body movements made with ease and without use of hands ; abduction of thighs causes very slight pain, and all pressure pains have disappeared or are diminishing ; increase in weight since admission of 3 kg. In general a marked improvement in patient's condition.

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THE EFFECTS OF RESPIRATION OF OXYGEN ON
BREATHING AND CIRCULATION. BY L. DAUTRE-
BANDE, M.D. (Louvain), AND J. S. HALDANE, M.D., F.R.S.

(From the Laboratory, Cherwell, Oxford, and the Lister
Institute, London.)

IT was shown by Paul Bert⁽¹⁾ that exposure of living organisms to an abnormally increased partial pressure of oxygen has, in general, a detrimental effect. In the case of warm-blooded animals, however, the effect which he discovered was only shown at oxygen pressures exceeding about three atmospheres. At pressures considerably higher than this the central nervous system was soon damaged irreparably, so that the animals did not recover. When removed from the atmosphere they remained comatose, and displayed symptoms similar in many respects to those of strychnine poisoning, and resembling also the after-effects of prolonged exposure to want of oxygen, as in CO poisoning. His description of the spastic condition of the limbs and easily induced opisthotonus in the animals experimented on corresponds rather strikingly with the similar symptoms recorded by one of us in miners who had been rescued alive after prolonged unconsciousness from CO produced in colliery explosions⁽²⁾.

Lorrain Smith⁽³⁾ discovered that though oxygen at less than three atmospheres' pressure produces in warm-blooded animals no evident nervous symptoms, yet prolonged exposure to even as little as 70 p.c. of an atmosphere of oxygen (or 60 p.c. in the alveolar air) may produce fatal pneumonia. The lung epithelium is of course exposed to the full effects of the oxygen; and it can hardly be doubted that if the far more sensitive tissues of the central nervous system were really exposed to a considerably increased oxygen-pressure very evident nervous symptoms would be produced. As will be shown in a paper shortly to be published by Douglas and Haldane, the blood passing through the brain of man loses under normal conditions probably not more than about 2 c.c. of oxygen per 100 c.c. of blood, and the CO₂-pressure of the blood does not rise more than about 3 or 4 mm. The co-efficient of absorption of oxygen in blood at body-temperature is .022. Hence when pure oxygen is breathed at two atmospheres pressure there will be in simple solution in the arterial blood, allowing for the presence of about 6 p.c. of

$\text{CO}_2 + \text{H}_2\text{O}$ in the alveolar air, about 4.14 c.c. of oxygen per 100 c.c., or 3.9 c.c. in excess of what is normally present. Hence if the circulation rate remained normal, there would be about 2 c.c. of free oxygen per 100 c.c. of the venous blood returning from the brain, and the oxygen-pressure in the brain would therefore be extremely high.

The experiments now to be described were undertaken for the purpose of seeing whether any evidence could be obtained that the tissues of the nervous system are defended against the influence of the oxygen by diminution of the circulation through them, so that the excess of free oxygen in the arterial blood is used up as it passes through the capillaries, and thus cannot, except at extremely high pressures of oxygen, reach the tissues. The diminution in the circulation through the brain capillaries might easily be brought about by contraction of capillaries in the manner recently described by Krogh (4). If the circulation is diminished when oxygen is breathed it is evident that the pressure of CO_2 in the tissues must rise; and in the respiratory centre this rise of CO_2 -pressure will, other things being equal, imply rise of hydrogen ion concentration and consequent increase in breathing and fall of alveolar CO_2 -pressure. A very slight fall in alveolar CO_2 -pressure will, however, suffice to compensate for the rise in CO_2 -pressure in the tissues in consequence of a sufficient slowing down of the circulation to reduce the oxygen-pressure of the venous blood to normal. Complete compensation could not, however, be expected, since otherwise there would be no stimulus left to account for the slowing down of circulation.

The problem which we set ourselves to investigate, therefore, was whether there is any fall in alveolar CO_2 -pressure when oxygen at increased partial pressure is breathed. We also watched the pulse carefully, as any diminution in pulse-rate would serve as an index of slowing of the circulation. It was already known from the careful experiments of Parkinson (5), that an appreciable, though small, diminution in pulse-rate is produced by breathing pure oxygen. Most of our experiments were carried out at ordinary atmospheric pressure; but one series was at increased atmospheric pressure in the large steel chamber of the Lister Institute. We are much indebted to Professor C. J. Martin for personal help and facilities afforded to us for this series.

The experiments were made as follows: two large Douglas bags were filled respectively with pure oxygen and air. The subject then sat quietly and breathed from one of the bags through Rosling valves and a comfortable mouthpiece (with a nose-clip on the nose) for at least five minutes. Towards the end of this period the pulse was repeatedly counted, and

finally a sample of alveolar air, obtained about one second after the completion of an inspiration, was taken by the ordinary Haldane-Priestley method, and the CO_2 determined.

The mean results at ordinary atmospheric pressure were as follows:

	Alveolar CO_2 percentage		Pulse		Number of pairs of observations
	oxygen	air	oxygen	air	
L. D.	6.04	6.25	75.1	81.0	36
J. S. H.	5.25	5.42	78.0	82.7	3

The following results were obtained in a very careful series made on one day in the steel chamber at a total pressure of 2.08 atmospheres or, 1580 mm.

	Alveolar CO_2 percentage		Pulse		Number of pairs of observations
	oxygen	air	oxygen	air	
L. D.	2.46	2.69	76.5	87.7	4

If we convert CO_2 percentages into pressures of CO_2 the results are as follows:

			Alveolar CO_2 -pressure in mm. of Hg.		
			Oxygen	Air	Fall with oxygen
At ordinary atmospheric pressure ...	{	L. D.	42.8	44.3	1.5
		J. S. H.	37.2	38.4	1.2
At 2.08 atmospheres	L. D.	37.7	41.2	3.5

Out of the 43 pairs of observations the CO_2 -pressure was only in six cases lower with air than with oxygen; and only in four cases was the pulse-rate higher with oxygen.

It is clear from these observations that when oxygen is breathed at normal barometric pressure there is a drop of about 1.5 mm. in alveolar CO_2 -pressure, and about five beats per minute in the pulse during rest sitting. At 2.08 atmospheres the drop is about 3.5 mm. in alveolar CO_2 -pressure, and eleven beats per minute. The experiments therefore confirm the theory (which is in itself probable from many considerations into which we need not enter here) that excess of free oxygen in the arterial blood causes slowing of the circulation. The result of this slowing down will be that in accordance with the conception running through so much of Claude Bernard's work, the "internal environment" of the tissues tends to be kept approximately constant in spite of great variations in the external environment. We can thus understand why it is that so high a pressure of oxygen is needed before the central nervous system is affected. The observed drop of 3.5 mm. in the alveolar CO_2 -pressure when oxygen at 2.08 atmospheres pressure was breathed would

correspond to a slowing in the brain circulation sufficient to reduce the oxygen-pressure in the blood leaving the brain to normal. An oxygen-pressure such as we tested in the steel chamber is sufficient to cause fatal pneumonia within two or three hours. Neither of us, however, could detect any definite subjective symptoms of psychical disturbance on breathing the oxygen for a few minutes. On the other hand distinct symptoms of irritation of the air-passages were produced in L. D., and did not pass off for several hours.

Our attention was directed to a paper by Yamada⁽⁶⁾ in which his alveolar CO₂-pressure was carefully measured in order to investigate the effects of breathing mixtures of oxygen, air, and CO₂. These experiments showed that whether or not CO₂ was present in the inspired air the alveolar CO₂-pressure was distinctly lower with oxygen than with air. He concluded that the oxygen increased the excitability of the respiratory centre to CO₂. This is, of course, a possible explanation, but is not probable in view of the evidence that want of oxygen increases the excitability of the centre to CO₂, or contributes towards exciting it⁽⁸⁾. In any case the experimental results of Yamada are in complete harmony with our own.

CONCLUSION.

Respiration of oxygen, particularly at increased barometric pressure, increases the breathing and diminishes the pulse-rate. These effects are presumably brought about by slowing of the blood-flow through the tissues, which protects them against the poisonous action of the high oxygen-pressure.

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STUDIES OF THE NUTRITIVE VALUE OF THE EDIBLE OILS AND FATS.

I.—The Oil-bearing Seeds and Crude Vegetable Oils and Fats.

By J. C. DRUMMOND, D.Sc., F.I.C., AND
S. S. ZILVA, D.Sc., Ph.D., F.I.C.

(From the Biochemical Laboratory, Institute of Physiology, University College, London, and the Biochemical Dept., Lister Institute, London.)

Very shortly after the recognition of the importance of the vitamins in nutrition it was shown that the nutritive value of edible oils and fats cannot be estimated completely in terms of calories, but that their value as foodstuffs may be greatly influenced by the presence or absence of one of these accessory food factors, namely vitamin A.

The earlier investigations tended to show that the fats of animal origin are valuable sources of this important dietary unit whilst those of vegetable origin are deficient in this respect. (For a complete review of earlier literature see Report on Vitamins published by the Medical Research Council. Special Report No. 38, H.M. Stationery Office, 1919.)

The important bearing of these observations on the question of the food value of butter substitutes was pointed out by Halliburton and Drummond (*J. Physiol.*, 1917, 51, 235), who confirmed the low vitamin value of the chief edible vegetable oils, and showed that butter substitutes prepared on an animal fat basis are nutritively superior to those compounded from vegetable sources.

This broad difference in the value of the two main classes of edible oils has been repeatedly confirmed in recent years, and certain of the later researches have yielded results throwing some light on the underlying causes.

It has been pointed out by Drummond and Coward (*Biochem. J.*, 1920, 14, 668) that no hard and fast line can be drawn between the animal and vegetable oils and fats when their value as a source of vitamin A is being considered. The chief factor controlling the amount of vitamin A in an animal fat is apparently the amount of that substance present in the diet which the animal has been consuming. A good example of this is provided by the experiments of Drummond, Golding, Zilva, and Coward (*Biochem. J.*, 1920, 14, 742), which provided a satisfactory solution of the problem why one animal fat, lard, is usually deficient in the factor "A."

The experimental results recorded in this paper represent one section of an exhaustive inquiry into the nutritive value of the chief edible oils and fats which we are conducting on behalf of the Medical Research Council, and deal with our attempts to explain the lower food value of the majority of vegetable oils.

At the outset of our inquiry we were of the opinion that the investigation would be particularly difficult, owing to the apparent necessity of tracing the fate of any associated vitamin A during the many processes through which vegetable oils pass before they are placed on the market as edible products. It must be remembered that in practically all the previous experiments which led to the vegetable oils being considered poor sources of vitamins, refined market products were employed. It was therefore obvious that in order to gain definite

information whether vegetable oils can be prepared containing appreciable amounts of vitamin A the richness of the raw materials in this factor must first be studied.

Accordingly we opened our investigation by an examination of the chief oil-bearing seeds used at the present time for the preparation of edible oils and fats. We were prepared to find that they possessed in general a low vitamin A content from the observations of McCollum and his many co-workers, who showed that the majority of the storage organs of plants, particularly seeds, contain comparatively little of that substance (McCollum, "The Newer Knowledge of Nutrition," New York, Macmillan, 1919), and also from certain experiences of our own with fat-rich nuts (Drummond and Coward, *Biochem. J.*, 1920, 14, 667).

The testing of the oil-bearing seeds presented some difficulties. In general the technique followed was that described by Drummond and Coward (*Biochem. J.*, 1920, 14, 661), the supplements of seeds being given separately from and before the daily ration of diet deficient in vitamin A. In certain cases in which the animals were not disposed to consume the seeds it was found necessary to incorporate the material with the basal diet, and this procedure was also necessary when a high daily intake of the seeds was required. In the latter case, where a considerable proportion of the daily ration was replaced by seeds, it was necessary to take into account the composition of the seed and the biological value of its constituent proteins in order to guard against the preparation of a badly balanced ration. Supplies of the seeds were obtained from several sources, and in this respect we wish to express our sincere thanks for the invaluable assistance we have received from Mr. J. Hanley, F.I.C., of Messrs. Bibbys, Liverpool, Mr. Dujardin, Olympia Mills, Selby, and the Director of the Imperial Institute, South Kensington.

Table I. gives a list of the oil-bearing seeds examined, their analysis, and their influence on growth when supplied as the sole source of vitamin A in the diet of rats.

Many difficulties stand in the way of a satisfactory interpretation of the results of feeding animals on complex foodstuffs such as these seeds. Certain of these seeds are not used directly as foodstuffs but only as the source of edible oils, and some actually contain substances injurious to the animal organism; such, for example, as the members of the cottonseed group, which may prove rapidly fatal to some species, including the rat. In other cases it was found unwise to grind the seeds before administering them to the animals in order to prevent consequent enzyme action producing undesirable changes.

It is, therefore, not always sound to conclude that a failure to resume growth on adding a supplement of seed to the basal diet is demonstrative of

an absence of the vitamin, unless other inhibitive factors can be excluded. When the seeds were known to be edible, or in cases in which the animals consumed the seeds with avidity over considerable periods without apparent ill effects, with little or no

temperature and pressure, the last traces being removed by passage of a rapid current of carbon dioxide at 80° C., in order to prevent loss of vitamin by oxidation. The prepared oils were tested by animal feeding experiments in the usual manner.

TABLE I.

Oil-bearing seed.	Origin.	Analysis.			Vitamin tests.		
		Moisture.	Fat.	Protein.	Approximate daily supplement in g.	Effect on growth and health.	Approximate value as source of vitamin A.
Linseed	Plate River 1 ..	10.6	22.7	21.9	{ 2 4	Practically no growth.	Fairly good.
Linseed	" " 2 ..	7.0	22.2	19.4		Slow but steady growth.	
Palm kernels ..	West Africa 1 ..	9.8	43.6	13.1	{ 6 2	Fairly good growth and health	Small.
Palm kernels ..	" " 2 ..	9.1	47.6	12.8		No growth but health good.	
Soya beans ..	Manchuria 1 ..	13.2	8.5	40.7	{ 4 6	Slight and irregular growth.	Nil.
Soya beans ..	" " 2 ..	10.2	6.8	—		Slow but steady growth.	
Soya beans ..	Russia	10.9	5.7	—	{ 2-6 2-4	Health fairly good.	Apparently toxic.
Cottonseed ..	Japan	11.9	10.1	—		No growth, health good, very slight growth on higher intake.	
Cottonseed ..	Bombay	11.9	10.1	—	{ 6 2-4	No growth. Death within few days.	Very low.
Cottonseed ..	Egypt	11.9	12.0	—		Very slight growth, death.	
Cottonseed ..	Upper Egypt ..	8.5	25.8	—	{ 2-4 4-6	No growth, health good.	Low.
Arachis	W. Coast	6.5	44.3	31.2		Slight growth.	
Rape	Toria	7.2	39.3	25.1	{ 2-4 2	Very slight growth, health good.	—
Fennel	Mediterranean ..	12.2	16.6	22.2		No growth, seeds apparently unpalatable.	
Babassu		4.4	63.7	—	{ 1-2 4-6	No growth and condition poor.	Very low.
Kapok		11.7	13.5	—		Slight but steady growth.	
Cohune	Ceylon	10.0	69.4	—	{ 2-4 2-4	Death after few days.	Toxic.
Djave	Brazil	4.8	64.6	—		No growth, health maintained, larger supplements not eaten.	
Djave	Gold Coast 1 ..	6.4	56.8	10.0	{ 2 2	Seeds not eaten well after first few days, no growth.	Nil.
Citician	W. Coast 2 ..	7.6	15.1	9.7		No growth, decline in health.	
Rangoon beans ..	Burma	11.5	4.3	23.9	{ 4 2	No growth.	Low.
Cacao	" "	7.4	38.8	—		Not eaten much. No growth.	
Candle nuts ..	Fiji	4.9	63.6	—	{ 2-4 3	Very slight growth.	Low.
Sesamé	Levant	—	48.2	—		Very slight growth, health good.	
Copra	W. Coast	—	62.0	—	{ 3 3	No growth.	Nil.
Maize (yellow) ..	U.S.A.	—	—	—		Slight growth.	
Maize (white) ..	S. Africa	—	—	—	{ 3 3	Practically no growth.	Nil.

resumption of growth, it was assumed with reason that the continued inhibition was due to a deficiency of vitamin A. Later results with certain of the crude extracted oils appeared to justify this assumption.

It will be seen from Table I. that of the many seeds tested only linseed had any appreciable value as a source of vitamin A. McCollum (J. Amer. Med. Assoc., 1917, 68, 1379) has also reported that linseed ranks higher than the majority of seeds in its content of vitamin A, but is not as valuable in this respect as millet. He examined wheat, corn, rice, oats, rye, barley, Kaffir corn, millet, flaxseed, peas, navy beans, and soya (see "The Newer Knowledge of Nutrition," loc. cit.).

Our results, therefore, tend to confirm the view that the plant transfers relatively small amounts of the unidentified fat-soluble vitamin A from the leaves where it is synthesised (Coward and Drummond, Biochem. J., 1921, 15, 530) to the reserve supplies of the seed, even when these are rich in fat.

On these grounds it would appear improbable that any oils or fats prepared from seeds will be found to possess a nutritive value approaching that associated with most animal fats such as butter or the fish and fish-liver oils. Nevertheless, we decided to investigate a few of the crude oils prepared from the seeds, particularly the most promising one, linseed, in order to exclude any inhibitive effect on growth which might have been due to other constituents of the seeds. The oils were mostly prepared in the laboratory from the freshly crushed seeds by cold extraction with frequent changes of petroleum spirit (b.p. 40°—60° C.). The extracts were rendered free from solvent at low

TABLE II.

Crude oils.	Approximate daily supplement.	Effect on growth.
Palm kerne	{ 1 ..	Very slight growth.
	{ 2 ..	Slow growth.
Soya	{ 1 ..	Very slight growth.
	{ 2 ..	Slow growth.
Arachis	{ 1 ..	Slight growth.
	{ 2 ..	Slow growth.
Linseed	{ 1 ..	No growth.
	{ 2 ..	Slight growth.
Djave	{ 1 ..	No growth.
Maize (yellow) ..	{ 2 ..	Very slight growth.
Rape	{ 2 ..	Slow growth.
Average butter ..	0.2 ..	Good growth.
" cod liver oil ..	0.02 ..	Good growth.

The majority of these results agreed with our expectations in that the small amount of vitamin in the seeds would presumably be carried over into the extracted oils. They also confirmed our previous observation that certain crude commercial oils may contain appreciable although low concentrations of the growth-promoting factor. Comparable figures for average samples of butter and cod-liver oil are also given.

The results obtained with the extracted linseed oil were, however, surprising, since we had found a diet supplying approximately 6 g. daily of the seed itself to be adequate for sustained although somewhat subnormal growth. This quantity corresponds to approximately 1.3 g. of oil, whereas the feeding experiments with the oil extracted from the same sample of seed showed a very decidedly slower rate of growth when 2 g. daily was supplied.

Destruction of the associated vitamin during preparation was unlikely by reason of the care with

which oxidative changes were guarded against, so that it would at first sight appear that the whole of the vitamin A in linseed is not present in a free condition in which it is extractable by fat solvents. The low vitamin A value of commercial refined linseed oil has been reported by more than one observer, but this oil is usually prepared by expression. We thought it of interest, therefore, to compare the nutritive value of two samples of linseed oil prepared from the same meal by the two chief processes, expression and extraction. Samples of these unrefined oils were placed at our disposal by Mr. Hanley and were tested in the usual manner, but were both found to be equally poor sources of the vitamin, only slow growth being obtained with supplements of approximately 2 g. daily. In the absence of more information from a larger number of experiments on quantitative lines, for which we have not yet had a suitable opportunity, the question must be left an open one.

It was our original intention to study the influence of the methods of neutralisation, deodorisation, and decolorisation of vegetable oils on their content of vitamin A, but our demonstration of the low value of the crude oils rendered such an inquiry of great difficulty and of little practical value. We are, therefore, proposing to postpone our investigation of that aspect of the refining of oils until we deal with the more highly potent oils of other types.

Of considerable interest in a discussion of the vitamin A value of vegetable oils is the case of crude palm oil. The curiously high value of this oil as a source of this dietary factor was observed some time ago by Drummond and Coward (*Biochem. J.*, 1920, 14, 671) and has been confirmed by us in the examination of several specimens from various sources. The more highly pigmented samples appear to show the higher growth-promoting activity in the feeding tests, but we are uncertain whether this is actually the case. More than one sample has possessed as high a potency as that exhibited by average samples of butter (*i.e.*, daily ration of 0.2–0.4 g. promotes growth in a 100-g. rat.).

The difference between the two oils derived from the fruit of the African oil palm (*Elais guineensis*) is most striking, and recalls the suggestion advanced by Steenbock (*Science*, 1919, 50, 352) that the vitamin A is associated with pigments of the lipochrome class. As is well known, crude palm oil derived from the fruit pulp of the African oil palm is deeply coloured with carotene and xanthophyll, the chief members of this group of natural pigments, whereas the oil derived from the kernel is almost colourless.

Whilst this theory has been found faulty in its general application (Palmer, Palmer, and Kempster, *J. Biol. Chem.*, 1919, 39, 299, 313, 331; Drummond and Coward, *Biochem. J.*, 1920, 14, 668), it is nevertheless true, especially of many vegetable products, that vitamin A is frequently found in association with these colouring matters.

The main object of these experiments was to seek a cheap source of vitamin A in the form of a vegetable oil suitable for margarine manufacture. Our results show that, with the exception of palm oil, none of the oils we have examined would be of any value in raising the nutritive value of vegetable oil butter substitutes.

If it were possible to obtain palm oil in a palatable form suitable for inclusion in such products, it would not be difficult to raise their vitamin value, but the difficulties of so purifying this oil without at the same time causing loss of the valuable accessory substance appear to be very great. One path of approaching this problem seemed to us to be by taking advantage of the fact that the whole of the vitamin A associated with fats may be obtained in the unsaponifiable fraction if care is taken throughout the process to exclude oxidation (Steenbock and Boutwell, *J. Biol. Chem.*, 1920, 42, 131; Drummond and Coward, *Lancet*, 1921, 11, 698). Accordingly we prepared fractions of the unsaponifiable matter of palm oil but found this product to possess to a marked extent the characteristic odour and taste of the original palm oil, so that without further treatment it would be quite unsuitable for inclusion in any appreciable proportion in vegetable oils for margarine manufacture. We have not yet had the opportunity to study whether this fraction can be converted into an edible product by processes sufficiently cheap to make the enrichment of vegetable oil margarines practicable by this means.

In conclusion, we wish to express our appreciation of the valuable assistance of Miss K. H. Coward, M.Sc., and Miss Low, and to acknowledge the financial grant from the Medical Research Council which enabled the investigation to be made.

Conclusions.

1. In order to investigate the low value of the majority of vegetable oils as sources of vitamin A, an exhaustive study of these oils and their raw materials was planned.

2. The present communication deals mainly with the examination of the chief oil-bearing seeds, which were found to be generally of very low vitamin A value, with the one exception of linseed.

3. An examination of certain crude oils prepared by extraction with petroleum spirit showed that the majority of the vitamin in the seeds passes into the oils, producing oils of very low potency as compared with the chief animal oils and fats. In the case of linseed the oil does not appear to contain the whole of the vitamin in the seed. It has not been yet investigated whether this is due to incomplete extraction or to loss by oxidative changes.

4. Crude palm oil may contain relatively high concentrations of vitamin A. An attempt to prepare from this oil a fraction consisting of unsaponifiable constituents suitable for raising the vitamin value of vegetable oil margarines was unsuccessful, since the product, whilst highly potent from a vitamin standpoint, was unpalatable.

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XXXI. THE RELATION OF THE FAT SOLUBLE FACTOR TO RICKETS AND GROWTH IN PIGS. II.

BY JOHN GOLDING, SYLVESTER SOLOMON ZILVA, JACK CECIL DRUMMOND AND KATHARINE HOPE COWARD (*Beit Memorial Research Fellow*).

From the National Institute for Research in Dairying, Reading, the Biochemical Department, Lister Institute, the Institute of Physiology, University College, London.

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THE experiments described in this communication form a part of an inquiry now in progress, the main purpose of which is to study the part played by the accessory factors in the nutrition of agricultural stock and in the etiological factors concerned in the causation of rickets in pigs. In a previous communication [Zilva, Golding, Drummond and Coward, 1921] in which the etiology of rickets in pigs was investigated we have shown that a rigorous elimination of vitamin *A* from the diet of young pigs did not conduce to the production of well-defined rickets. We were, however, able to demonstrate in those experiments that such a dietetic deficiency has a very marked effect on the development of these animals. Indications were also obtained that the deprivation of this dietetic principle possibly has some bearing on the production of healthy young.

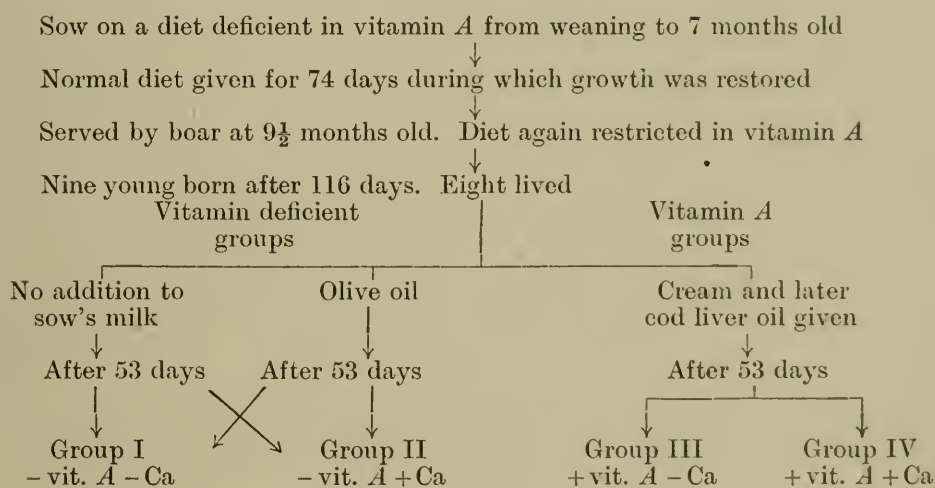
Having been unsuccessful in producing rickets in pigs experimentally by depriving them of vitamin *A* alone we next attempted to ascertain whether a dietetic deprivation of calcium and vitamin *A* would lead to the production of the disease. The results obtained by us form the subject of this communication.

EXPERIMENTAL.

Our animals were divided into four groups. Group I received a diet deficient in vitamin *A* and in calcium ($-A - Ca$), group II received a diet deficient in vitamin *A* ($-A + Ca$), group III received a diet deficient in calcium only ($+A - Ca$), group IV received a diet containing calcium and vitamin *A* ($+A + Ca$). Two pigs were placed in each group. Group I consisted of two boars, group II of one boar and one sow, group III of two sows and group IV of two boars. The young pigs, which belonged to the same

litter, were started on their special diets when they were 53 days old. They were young Berkshires, born of a sow 14 months old. The sow was kept for some time on a diet of toppings and whey deficient in vitamin *A* and manifested this deprivation by retarded growth [Drummond, Golding, Zilva and Coward, 1920]. On supplementing the above diet with lucerne the animal resumed growth and continued an apparently normal existence. After 74 days of correct feeding she was served by a pedigree boar and soon after was placed again on a diet of toppings, whey and swedes. This diet which was poor in vitamin *A* was purposely planned as we did not desire the young pigs to be born with a store of the vitamin. After 116 days nine pigs were born and at the age of 65 hours were divided into two sections. The chart below shows the history and subsequent allocation of the pigs. One lot received its supply of vitamin *A* entirely from the mother, the other received additional vitamin *A* in the form of cream ($\frac{1}{8}$ oz. per day) and eventually in the form of cod liver oil ($\frac{1}{2}$ rising to 1 oz. per day). Two of the animals in the former section received an equal supply of additional oil in the form of inactive olive oil, made into an artificial cream at first and later given to balance in nutrients the cod liver oil given to Section II.

The two remaining pigs in the first section received only the sow's milk; the sow being fed all the time on a diet shown to be poor in the vitamin *A*.



As soon as it was convenient the young pigs were given additional toppings which at the end of this preliminary period of 53 days (period I) reached a ration as high as 3 lbs. per day for the litter of eight pigs. The actual intake of food and the corresponding increase in weight for the entire experiment is summarised in Table I. The growth of the animals is graphically represented in Fig. 1. The average daily gains in pounds for period I were for pigs in section I receiving no oil 0.533 and 0.514, for those receiving olive oil 0.377 and 0.5, while the daily gains of the pigs in section II receiving cod liver oil were 0.481, 0.453, 0.344 and 0.509.

The agreement between the rates of growth of the pigs in the two sections is made more evident by employing the formula advised by R. A. Fisher,

Table I

[illegible]

Period V Groups I and II (a) Heated caseinogen	Weight on Sept. 29th Gain in 7 days Relative growth rate Toppings, dry matter Caseinogen Oil Charcoal Chalk Pounds dry matter in food per 1 lb. increase in live weight	64.75 - 0.5 Loss 10.2 0.62 H 0.52 O 0.44 W — —	70 1.75 0.36	82 0.5 0.09	114.25 6.0 0.77	99.5 6.25 0.92	125 8.5 1.0	1.42 12.75 1.34
		13.72 1.3 H 1.12 O 0.87 A 0.42 7.75			57.08 1.3 U 1.12 C 0.87 W — 4.92			58.41 1.3 U 1.12 C 0.87 A 0.43 2.92
(b) Unheated caseinogen	Weight on Oct. 2nd Gain in 3 days Relative growth rate Toppings, dry matter Caseinogen Oil Charcoal Chalk Pounds dry matter per 1 lb. increase in live weight	69 4.25 2.11 4.64 0.28 U 0.24 O 0.2 W — 1.26	71.5 1.5 0.7	86.5 4.5 1.78	116 1.75 0.51	101 1.5 0.50	130 5 1.31	145.5 3.5 0.81
		7.96 0.56 U 0.48 O 0.37 A 0.18 1.59			25.32 0.56 0.48 C 0.37 W — 8.23			26.55 0.56 U 0.48 C 0.37 A 0.18 3.31
(c) Heated caseinogen	Weight on Oct. 22nd Gain in 20 days Relative growth rate Topping's dry matter Caseinogen Oil Charcoal Chalk Pounds dry matter per 1 lb. increase in live weight	76.25 7.25 0.50 25.2 1.95 H — 1.3 W 3.9	80.75 9.25 0.61	93 6.5 0.36	135.5 19.5 0.776	120.25 18.25 0.87	162 32 1.10	167 21. 69
		57 3.9 H — 2.6 A 1.3 4.1			180 3.9 U — 2.6 W — 4.9			197 3.9 U — 2.6 A 1.3 3.8

* = Weight of Pig, group I on Aug. 25th. Caseinogen U = Unheated, H = Heated. Oil C = Cod Liver Oil, O = Inactive Olive Oil. Charcoal A = Animal Charcoal, W = Wood Charcoal.

NOTE. The total dry matter consumed is calculated on the ingredients given and expressed in pounds and decimals of a pound. The small daily dose of lemon juice and the occasional doses of marmite are set against loss of charcoal and chalk which were not always cleaned up.

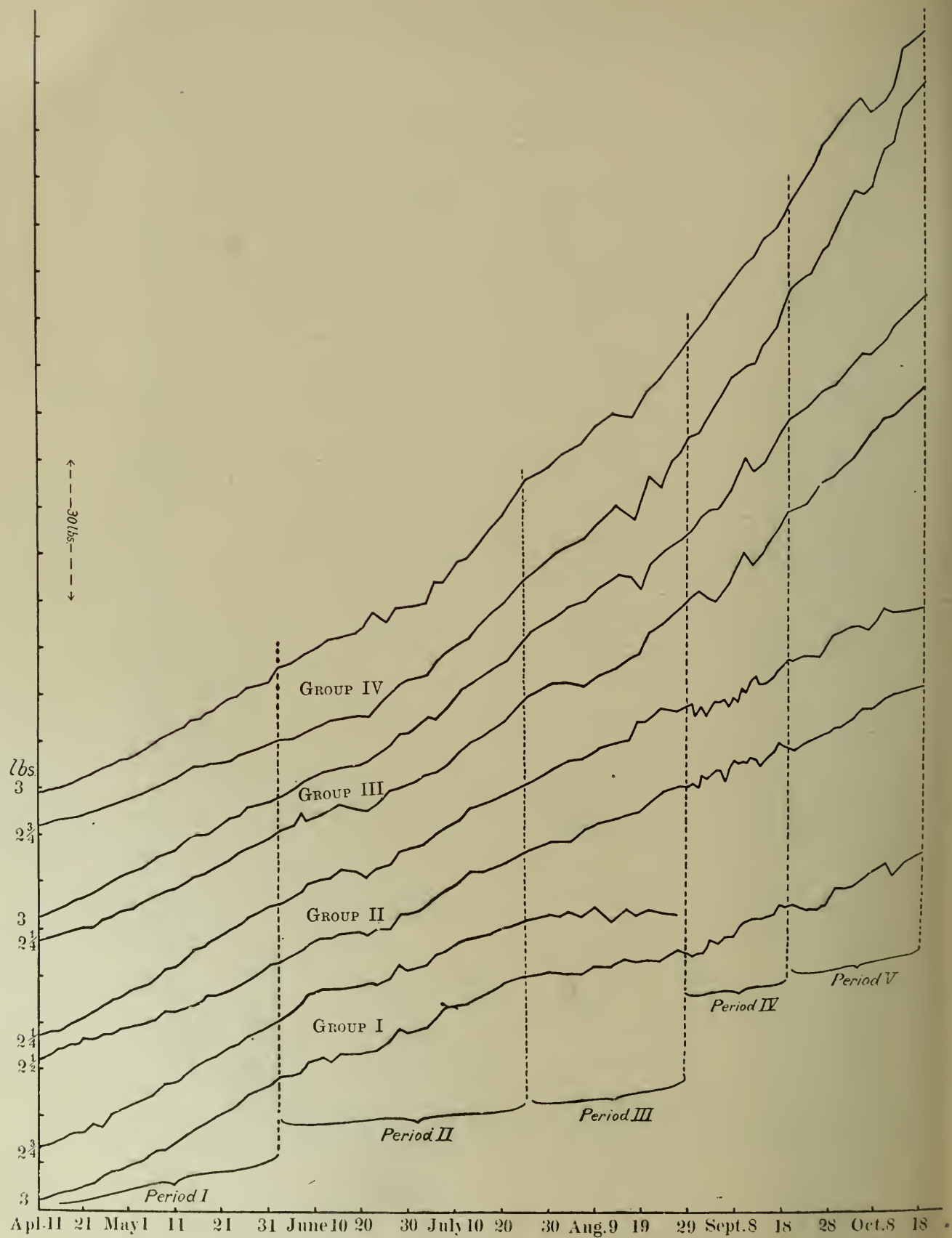


Fig. 1.

Chief Statistician, Rothamsted Experimental Station, for calculating the relative growth rate per day per cent. using natural logarithms, viz.:

$$\frac{\log_e M_2 - \log_e M_1}{K_2 - K_1} 100$$

where M_2 = the weight at the end of the period,

M_1 = „ „ commencement of the period,

$K_2 - K_1$ = duration of period in days.

The following figures are obtained for Section I ($-A$) without oil 4.91 and 4.50, with olive oil 4.14 and 4.31, for Section II ($+A$) 4.25, 4.45, 3.83 and 4.34.

It is evident from the above figures that a sow, even when fed on a diet deficient in the fat-soluble factor and having undergone a previous deprivation in this factor, is capable of rearing her young satisfactorily. This confirms further our earlier observation that the requirements of the pig for the fat-soluble factor are not of a high order.

At the end of this period, namely 53 days after birth, the animals were weaned and placed on their respective experimental diets. Each group was placed in a separate sty. The sties faced south and were divided by wooden partitions, the floor being partly wood and partly concrete. The bedding consisted of wood shavings or sawdust.

The basal diet for all groups consisted of toppings or wheat offal having the following average composition:

Moisture	11.51 %	Mucilage	59.60 %
Oil	4.29	Woody fibre	5.86
Protein	14.81	Ash	3.93

The dry matter of the above contained 0.338 % calcium. Besides the basal diet the pigs received supplementary protein in the form of caseinogen, which in the case of the vitamin-free diets was previously inactivated by being heated for 24 hours at 120° C. This inactivation was carried out for us by Dr R. T. Colgate in Messrs. Huntley and Palmer's Laboratory, for which we wish to express our thanks.

The food was weighed out three times a day at 8 a.m., 12 noon and 4.30 p.m.; it was mixed with cold water just before feeding and given in the form of a thin cream. The food was given on a live weight basis, being regulated to the quantity which the pigs would clean up.

The other two accessory factors, namely the antiscorbutic and the anti-neuritic factors, were supplied in the form of freshly prepared lemon juice and marmite (Commerical Yeast extract), about 7 cc. of the former being the daily dose, whilst $\frac{1}{8}$ oz. of the latter was given at intervals. Groups III ($+A - Ca$) and IV ($+A + Ca$) received a daily dose of cod liver oil as a source of the fat-soluble factor whilst the other two groups received an equivalent dose of inactive olive oil. Groups II and IV also received a daily dose of 1 oz. each of precipitated chalk and 1 oz. animal charcoal containing

67.3 per cent. of calcium phosphate as a source of additional calcium and phosphate; the other two groups received only the small amounts of calcium from the basal diet.

During the following 54 days (period II) the intake in all the four groups was the same. The increase in weight in the animals of the various groups however showed a marked disparity. The two animals in group I gained 41.88 lbs., in group II 49 lbs., in group III 62.7 lbs., and group IV 74.6 lbs. The relative growth rates, as calculated from the formula given above, were group I 0.99 and 0.98, group II 1.32 and 1.13, group III 1.47 and 1.34, and group IV 1.80 and 1.57. The average dry matter in the food consumed per pig for each pound of gain in live weight was: group I 4.4 lbs., group II 3.8 lbs., group III 3.0 lbs. and group IV, 2.5 lbs. By this time very marked differences in the general appearance of the animals in the respective groups could be discerned. The animals in group I developed a scurfy skin and saddle back, weak legs and joints painful to pressure. They were easily tired and were not playful. Those in group II also showed lack of vitality and a saddle back, those in group III were in good condition possessing glossy coat, whilst the animals in group IV were decidedly in the best condition. One animal in group I received an injury from a fall and died 87 days after the commencement of the experiment. At the post mortem examination it was found that the vertebral column was broken.

During period III, *i.e.* 35 days following period II, the increase in weight was as follows: group I 4.62 lbs. (one animal), group II 28.75 lbs., group III 42 lbs., and group IV 58.4 lbs., and the average dry matter in the food consumed per pig for each pound of gain in live weight was: group I (one animal) 14.6 lbs., group II 4.5 lbs., group III 4.0 lbs. and group IV 3.02 lbs.

Owing to the low condition of the remaining animal in group I it was decided to administer a small amount of the fat-soluble factor in order to save the pig. This was done by introducing caseinogen, which had not been previously inactivated by heating, in the diet during period IV. This addendum had its desired effect and the animal responded after about five days by resuming growth.

During this period of 22 days (period IV) the animals gained in weight as follows: group I (one animal) 10.25 lbs., group II 19 lbs., group III 41.7 lbs., and group IV 61.7 lbs. In the last period (period V) the inactivated caseinogen was alternated with crude caseinogen in order to keep the weight of the animals in group I and group II in check.

The experiment was terminated 145 days after its commencement. The animals were slaughtered with the exception of one sow in each of the groups II and III. The condition of the animals before slaughter was as follows: groups I and II wrinkled skin, ears carried forward,[§] down on hind legs; group III skin rather rough, lack of size and bloom, flesh not quite firm, otherwise normal; group IV skin healthy and animals perfectly normal.

The photographs of the pigs before slaughtering are shown in Plate II,

two photographs having been taken of the pig in group I. The photographs were taken to scale.

The post mortem examination revealed no abnormalities of the organs beyond that the ribs of the animals in group I were easily fractured. It is also to be reported that the fat of the pigs of groups I and II was, in the butcher's opinion, softer and not of such good quality as the fat of the other pigs. This was confirmed by the estimation of the melting and solidifying points of the fats.

Group		Melting points		Solidifying points
		I	II	
	I	21°	22.9°	19°
	II	21°	23.8°	19.3°
	III	25°	25.20°	22°
	IV	31.5°	32.8°	22.5°

Table II gives the size, weight, breaking points, and calcium content of the bones.

Table II.

Group		Weight of	Distance of	Breaking	Same	CaO
		humerus	bearing points	weight	corrected to	percentage of
		in g.	in inches	in tons	3½" length	dry matter
I	I	103	3½	0.210	0.210	29.52
II	II	127	3½	0.345	0.345	29.05
III	III	117	4	0.292	0.336	33.52
IV	IV	152	4	0.466	0.532	38.08

No abnormal flavour or taste was reported by a number of people who consumed the joints derived from the animals fed on the cod liver oil.

The fifth, sixth and seventh ribs of one animal in each group were examined for us histologically by Prof. V. Korenchevsky to whom we are also indebted for the interpretation of the results. The following is a summary of the observations made:

(1) Group IV (+ A + Ca) showed a somewhat abnormal picture with very slight osteoporosis and a belated deposition of lime salts in the newly formed bone.

(2) In all cases the bone marrow especially in the region of the secondary spongiosa consisted of a fine fibrous reticulum with but few bone marrow cells.

(3) The histological condition in animals belonging to groups II (− A + Ca) and III (+ A − Ca) was not very different from that of group IV (+ A + Ca). Only a higher degree of osteoporosis resulting from a diminished activity of osteoblasts could be seen.

(4) In group I (− A − Ca) the condition of osteoporosis was more pronounced. Moreover a more frequent incursion of the proliferating cartilage into the bone marrow was in evidence. In these places was also noticed defective calcification in the zone of provisional calcification.

It is quite evident that in spite of the very marked changes which have been effected by our restricted diets, no rickets in the pathological sense of the word has been induced. The animals in groups I and II have on various

occasions during the experiment displayed a condition which would have been described by the practical man as the pigs being "off their feet." No doubt such a condition has been before now loosely referred to as "rickets." Although defective calcification was found in the zone of provisional calcification in the case of group I ($-A - Ca$) no increase in the amount of osteoid tissue could be established and therefore no faulty deposition of calcium in the newly formed bone in the sense of rickets can be asserted.

A remarkable feature in our experiments is that even in the case of group IV ($+A + Ca$) which acted as our control, a normal histological picture was not obtained. This requires further investigation. Possibly the restricted diet of the mother may be responsible for this. Another point to be considered is that the animals consuming the calcium-deficient diet received 0.338 % of calcium in their food. With animals, such as pigs, which consume large bulks of food it was difficult from a technical point of view to reduce this calcium intake. However the calcium deficiency was definite enough to impair the growth and diminish the calcium content in the bones of the animals in group III ($-Ca + A$) and it is very doubtful whether pigs which develop rickets under farm conditions, receive a diet much poorer in this element. We refrain from reviewing the literature which has appeared during the last few months in connection with the etiology of rickets. Most of the experimental work was done on rats and the results and conclusions of the various investigations are conspicuously contradictory. We cannot however conclude without briefly referring to the results obtained by Korenchevsky [1921]. This investigator, working with rats, obtained a definite condition of rickets on a diet free from the fat-soluble factor and calcium. Whether our apparently different results were due to the higher content of calcium in our experimental diets, or whether it was due to the different character of the experimental animal will most probably be decided by future investigation. We are continuing our experiments and although the dietetic hypothesis of the etiology of rickets forms our main line of work, we are not excluding such a hypothetical factor as light, especially in view of the latest work of Hess and Unger [1921], of Powers, Park, Shipley, McCollum and Simmonds [1922].

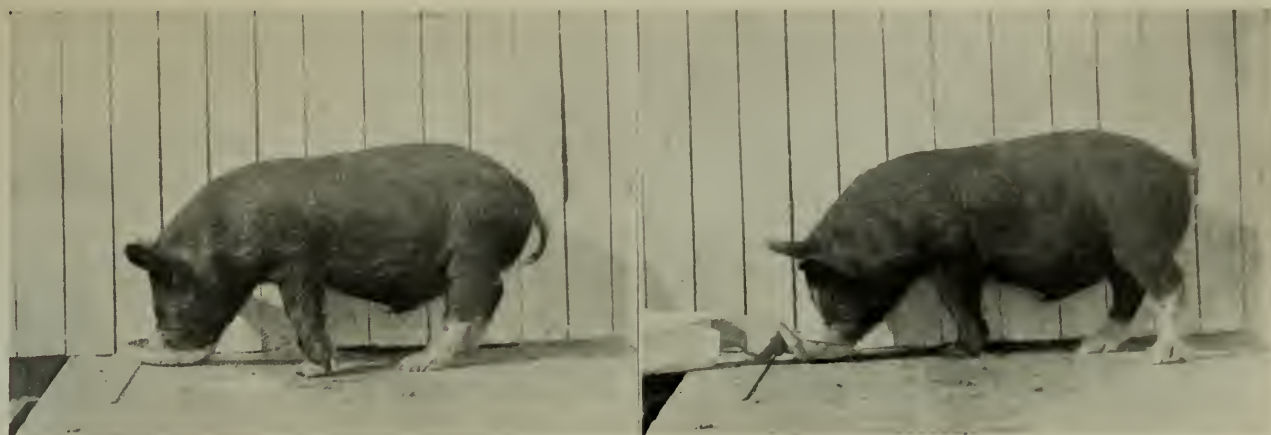
The expenses of this research were defrayed from a grant made by the Medical Research Council, to whom our thanks are due.

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PHOTOGRAPHS OF PIGS TAKEN ON OCT. 10TH, 1921

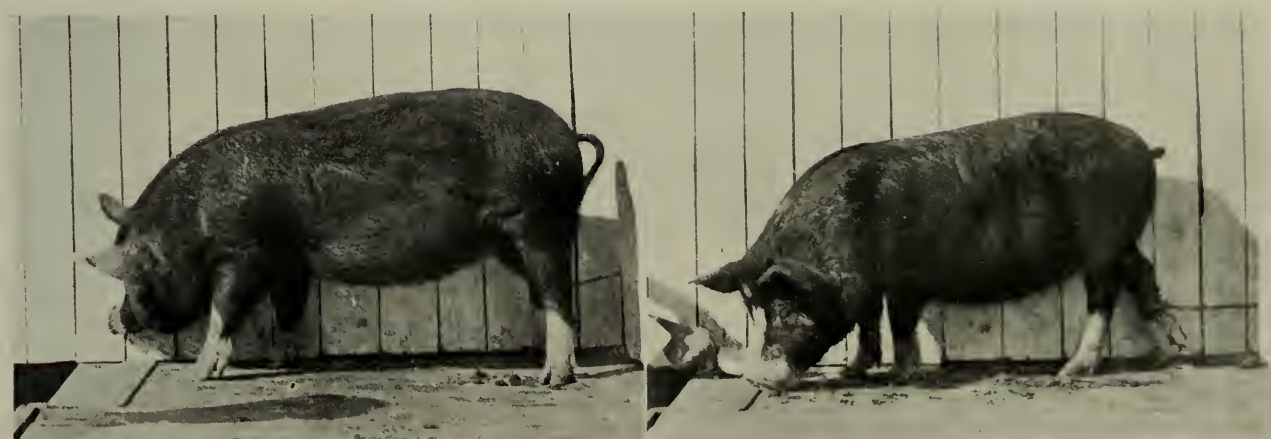
Group I - Vitamin A.



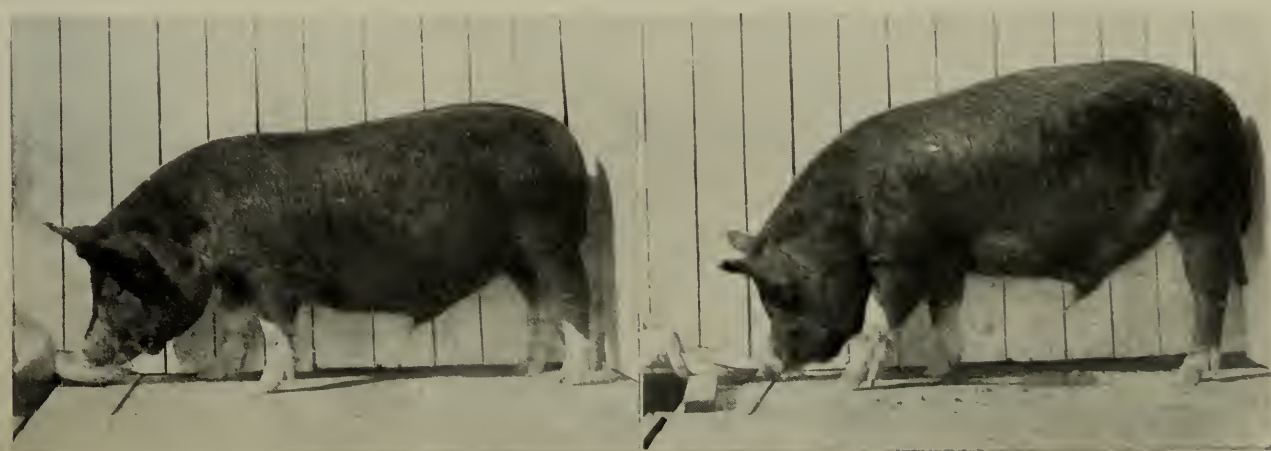
Group II - Vitamin A + Calcium and Phosphate.



Group III + Vitamin A.



Group IV + Vitamin A + Calcium and Phosphate.



N^o 7

XX. THE FUNCTION OF PHOSPHATES IN THE OXIDATION OF GLUCOSE BY HYDROGEN PEROXIDE.

BY ARTHUR HARDEN AND FRANCIS ROBERT HENLEY.

From the Biochemical Department, Lister Institute.

(Received January 25th, 1922.)

THE conditions under which the oxidation of glucose by H_2O_2 can take place in aqueous solution have been studied by W. Löb and his co-workers [1910, 1911, 1912, 1915]. He believes that the oxidation depends on the hydroxyl ion concentration of the solution, and further that phosphate ions exercise a specific accelerating effect on the reaction. He was unable to produce any oxidation of glucose by H_2O_2 in presence of water alone or when other substances were substituted for phosphates.

For instance neither Sørensen's mixtures of glycol and NaOH nor of borate and HCl could be used to replace the phosphate mixture. Witzemann [1920] confirmed Löb's observations and agreed that phosphate mixtures exercise a specific effect. He was unable to produce the same effect with mixtures of NaHCO_3 and Na_2CO_3 as with phosphate mixtures.

Witzeman [1920] suggested that the phosphates might produce an intermediate compound with glucose of the same nature as the hexosephosphate which has been shown by Harden and Young [1910] to be produced during alcoholic fermentation, and which appears from the work of Embden and others to be connected with the production of lactic acid in muscle.

Witzemann was unable to detect the formation of any compound of glucose and phosphate in absence of H_2O_2 .

As more exact knowledge of the mechanism of this reaction is desirable in view of the part played by phosphate in alcoholic fermentation, further experiments were made to try and ascertain whether an intermediate compound of the nature of a phosphoric ester of glucose was formed during this oxidation. For this purpose free phosphate was estimated at intervals in a mixture similar to one of those used by Witzemann for oxidation of glucose by H_2O_2 at 37° , both by the method of Schmitz [1906] and by the ordinary magnesium citrate method. No change in the amount of free phosphate was observed.

Besides the possibility of the formation of an intermediate compound between phosphate and glucose there are at least two other possible explanations of the effect produced by phosphate in this reaction:

(1) Phosphate may act as a peroxidase, thus rendering possible the oxidation of glucose by H_2O_2 . If this were so phosphate might act in the same way towards mixtures of H_2O_2 and benzidine or guaiacum. No evidence could be obtained of such an action, but it does not follow from this negative result that no such action occurs with H_2O_2 and glucose.

(2) The phosphate mixtures may simply act as buffer substances, the maintenance of the P_H within certain points being essential. If this hypothesis is correct it should be possible to replace the phosphate mixtures by other substances, provided the P_H be maintained within the same limits.

We have carried out experiments on the oxidation of glucose by H_2O_2 with solutions the P_H of which was never over 7.3 and find that glucose is oxidised by H_2O_2 in presence of 0.125 *M* NaHCO_3 saturated with CO_2 ; or 0.25 *M* sodium arsenate saturated with CO_2 ; or 0.25 *M* sodium acetate.

The phosphate ion cannot therefore be regarded as playing a specific part in the reaction. It appears more likely that the buffer action of the salts employed is the important factor, whether as providing and maintaining the most suitable conditions for oxidation or as protecting the H_2O_2 from too rapid decomposition. In this connection the experiment of Witzemann [1920, p. 12, sec. 7] with a mixture of sodium carbonate and bicarbonate should be referred to. This shows that in presence of these salts, at a P_H slightly over 9.3, H_2O_2 is rapidly decomposed and little oxidation takes place, whereas in our experiment in presence of 0.125 *M* NaHCO_3 saturated with CO_2 (P_H 6.8) a considerable amount of oxidation is produced.

Experiments were accordingly made roughly to test the stability of H_2O_2 in solutions the P_H of which was maintained at various levels by the use of buffer solutions. The rate of decomposition was found to increase with rise of alkalinity. In those cases in which the buffer solutions consisted of phosphate mixtures the rate of decomposition of the H_2O_2 was not so rapid as in the corresponding experiments with other buffer solutions; so that in this sense the phosphate ion may be said to have a specific action.

In the experiments of both Löb and Witzemann the P_H of the solutions was only carefully measured in those experiments in which Sørensen mixtures were used. In most if not all of the experiments in which the effects of other substances were compared to that of phosphate mixtures the reaction of the solution was tested with litmus paper only. The glycol + NaOH and borate + HCl mixtures used are only efficient buffers between P_H 8.0 and 10.2, and at these higher degrees of alkalinity the H_2O_2 is rapidly decomposed. The same applies to the $\text{NaHCO}_3 + \text{Na}_2\text{CO}_3$ mixture used by Witzemann which must have been much more alkaline than the phosphate mixture.

The experiments in which he compared the effect of Na_2CO_3 and NaOH were not carried out at the same P_H . The reaction of the solution in the latter case was suitable for the oxidation, whereas the alkalinity of the solution used in the former case was too high.

EXPERIMENTAL.

I. *Experiments to determine if mixtures of Na_2HPO_4 and NaH_2PO_4 will act as a peroxidase towards H_2O_2 and benzidine or guaiacum.*

Phosphate mixtures of P_H 9.0, 7.4, 6.9, 6.6, 6.2, 4.8 were tested with H_2O_2 and benzidine or guaiacum. No action was observed in any of the experiments.

To test whether the optimum P_H for the action of a peroxidase lies within the above range, similar experiments were made with the same phosphate mixtures, H_2O_2 and guaiacum with the addition of potato juice. The maximum effect was produced at room temperature from P_H 7.4 to 6.9. A similar result was obtained when benzidine was substituted for guaiacum.

II. *Oxidation of glucose by H_2O_2 in presence of various buffer mixtures.*

Seven flasks were made up as follows:

	Glucose	NaHCO_3	Na_2HAsO_4 12 H_2O	$\text{NaC}_2\text{H}_3\text{O}_2$ 3 H_2O	K_2HPO_4	KH_2PO_4	5.7 % sol. H_2O_2	
	g.	g.	g.	g.	g.	g.	cc.	
1	8.75	2.6	—	—	—	—	62	} to 250 cc. with distilled water
2	8.75	—	25.1	—	—	—	62	
3	8.75	—	—	8.5	—	—	62	
4	8.75	—	—	—	8.8	2.2	62	
5	11.25	2.6	—	—	—	—	—	
6	11.25	—	25.1	—	—	—	—	
7	11.25	—	—	8.5	—	—	—	

In Nos. 1, 2, 5, 6 the solution was saturated with CO_2 .

The P_H of each solution was estimated colorimetrically, and the glucose estimated by Bertrand's method and by the polarimeter.

All the flasks were then kept in the incubator at 37° for 24 hours and the P_H and glucose again estimated in each. The results are shown below:

	At start of experiment glucose g. per 100 cc.			After 24 hours			Apparent loss of glucose in g. per 100 cc.	
	P_H	Bertrand	Polarimeter	P	A	B	by A	by B
		A	B					
1	6.8	4.16	3.50	6.7-6.8	3.39	2.99	0.77	0.51
2	6.8	3.96	3.55	6.7-6.8	2.96	2.38	1.00	1.17
3	6.8-7.0	3.64	3.54	5.2	3.01	2.50	0.63	0.53
4	7.0	3.65	3.20	5.6-5.8	2.24	1.85	1.41	1.35
5	6.8	—	4.55	7.6	—	4.55	—	0
6	6.8	—	4.50	7.0	—	4.50	—	0
7	7.3	—	4.25	7.3	—	4.25	—	0

Note. The sample of glucose employed for these experiments showed a 5 % higher content of glucose as estimated by Bertrand's method than when the polarimeter and $[\alpha]_D$ 52.5° were used. This, and the fact that the Bertrand estimations of glucose at the start of the experiment were carried out before the polarimeter readings were made, may account for the discrepancies between the two sets of figures shown above.

Only traces of H_2O_2 remained in 1, 2 and 4 after 24 hours in the incubator.

No. 3 still contained a considerable amount of H_2O_2 and the action was allowed to proceed for a second period of 24 hours. After this the P_H was

found to be 4.6–4.8 and glucose estimated polarimetrically amounted to 1.76 g. per 100 cc.: a total apparent loss of glucose of 1.78 g. per 100 cc.

These experiments show that in absence of H_2O_2 no loss of glucose has apparently taken place (Nos. 5, 6, 7). Whereas in presence of H_2O_2 (Nos. 1, 2, 3, 4) an apparent loss of glucose has occurred in every case, greatest in the two solutions containing phosphates and arsenates, but considerable in the other two solutions.

III. *Effect of change of P_{H} on the stability of H_2O_2 .*

All solutions contained 5.0 cc. of 5.7 % H_2O_2 per 100 cc. solution. The H_2O_2 was estimated at the start and again after 24 hours and 48 hours at 37° by titration with standard permanganate.

No.	Mixture	P_{H}	% H_2O_2 lost	
			after 24 hours	after 48 hours
1	HCl	1.0	—	1
2	Sørensen's glycol + HCl ...	1.9	1	2.1
3	Na acetate + acetic acid ...	4.6	2	2.1
4	Phosphates	6.8	2	4.2
5	"	7.1*	4.9	7.5 (43 hours)
6	"	7.8*	6.1	7.4 (43 hours)
7	"	8.0*	7.5	13.1
8	Palitzsch's borax + boric acid	6.9	20.2	36.1
9	" " "	7.1	20.2	37.2
10	" " "	7.7	22.3	39.3
11	" " "	7.9	37.2	59.5
12	" " "	8.2	55.3	74.4
13	" " "	8.4	65.9	84.0
14	" " "	8.6	65.9	84.0
15	" " "	8.9	67.0	85.1
16	Sørensen's borate + HCl ...	8.8	—	78.5
17	" + NaOH ...	9.2	—	99.0
18	" " ...	12.8	100	—

* In these cases the concentration of the hydrogen peroxide used was 5 % instead of 5.7 %.

IV. *The P_{H} of some of the solutions tested by Witzemann.*

A solution was made up as described by Witzemann [1920, p. 12, sec. 7]: 2.43 g. $\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$; 0.72 g. NaHCO_3 ; 35 cc. H_2O ; 20 cc. (0.2 g.) glucose solution and 20 cc. of 3 % H_2O_2 .

The P_{H} , estimated colorimetrically, was over 9.3. At this degree of alkalinity H_2O_2 is rapidly decomposed and it is therefore not surprising that little oxidation took place.

To compare the effect of NaOH and Na_2CO_3 Witzemann [1920, p. 14, sec. 9 (2) and sec. 10 (2)] prepared solutions as follows:

P. 14, sec. 9: 32.0 cc. 0.33 M Na_2HPO_4 ; 3.0 cc. H_2O ; 20.0 cc. 1 % glucose; 20.0 cc. 3 % H_2O_2 ; 0.61 g. $\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$;
and sec. 10 (3) 25.6 cc. 0.33 M Na_2HPO_4 ; 6.4 cc. 0.33 M NaH_2PO_4 ; 20.0 cc. 1 % glucose; 20.0 cc. 3 % H_2O_2 ; 3 cc. H_2O ; 0.1714 g. NaOH.

The P_{H} of the former was 9.3–9.6 and of the latter 7.1. The amount of oxidation was greater in the second than in the first case and Witzemann argues that NaOH exercises a less harmful effect on the reaction than does Na_2CO_3 .

It does not seem that this inference can fairly be drawn unless the experiments are carried out under the same conditions of P_{H} . The acidity of the H_2O_2 solution used by Witzemann is not stated in his paper. It is therefore not possible to say what was the exact P_{H} in his experiments. But in any case the alkalinity of the solutions used in experiments 7 and 9 (2) must have been much higher than in experiment 10 (3).

SUMMARY.

1. The oxidation of glucose by H_2O_2 takes place in presence of the following buffer substances: $\text{NaHCO}_3 + \text{CO}_2$; $\text{Na}_2\text{HAsO}_4 + \text{NaH}_2\text{AsO}_4$; $\text{NaC}_2\text{H}_3\text{O}_2$; $\text{K}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$, provided the P_{H} does not rise much above 7.3.
2. The stability of H_2O_2 in aqueous solution is increased by the presence of phosphates which do not however show a specific action in other respects.

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LXIV. THE ANTI-SCORBUTIC PROPERTIES OF CONCENTRATED FRUIT JUICES. PART IV.

By ARTHUR HARDEN AND ROBERT ROBISON.

(Received July 27th, 1921.)

IN earlier communications [1919, 1920] we have shown that orange juice can be evaporated to a dry residue without suffering any loss of the anti-scorbutic accessory factor, and that the product retains its potency in a very considerable degree after two years' storage in a dry atmosphere at room temperature.

The effect of storage at higher temperatures (29° C.) has now been investigated and, from the results obtained, it is evident that the accessory factor is much more rapidly destroyed under such conditions.

This particular sample of orange juice was dried in December 1919 and was found to afford complete protection from scurvy when fed to guinea-pigs in daily doses of 0.15 g., equivalent to 1.5 cc. of the original juice. The very hygroscopic solid was transferred as rapidly as possible to a screw-top glass jar, which was then placed in a desiccator for a few days before being closed down. In spite of these precautions it is probable that the material absorbed some moisture, for on placing the jar in the incubator, slight softening was seen to take place and the top layer of the substance gradually darkened in colour, owing presumably to oxidation. The jar was kept at a uniform temperature of 29° until February 1921, *i.e.* during 14 months, after which further animal tests were begun. The material was then in the form of a hard toffee-like mass but was found to contain 8 % of water.

A number of guinea-pigs were fed on the usual basal ration of oats, bran and autoclaved milk, together with doses of the dried juice of 0.15 g., 0.3 g., 0.45 g., and 1 g., equivalent to 1.5 cc., 3 cc., 4.5 cc., and 10 cc. of the original juice. All the animals developed scurvy but those receiving the highest dose did not show any symptoms until the 24th day and were still alive on the 63rd day of the experiment. Some measure of protection was therefore given by this amount of the dried juice, but it is evident that more than 85 % of the anti-scorbutic accessory factor had been destroyed during storage under the above conditions. Tests were also carried out with some of the same batch of dried juice that had been stored in a desiccator at room temperature during a similar period, 14 months. Two guinea-pigs, each receiving 0.15 g. daily, showed normal growth for about 50 days but thereafter remained practically stationary in weight. They were killed on the 81st day and symptoms of scurvy were found. A guinea-pig which received a daily ration of 0.3 g.,

equivalent to 3 cc. of juice, showed no signs of scurvy after 81 days, though this animal also failed to exhibit normal growth during the last 20 days of the experiment.

In the experiments previously described it was found that a daily ration of 0.5 g. of dried juice that had been stored during two years at room temperature afforded complete protection from scurvy. It would appear, therefore, that under the conditions detailed above the loss of the anti-scorbutic accessory factor is of the order of 50 % of the amount originally present in the juice.

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No 9

HUNGER - OSTEOMALACIA IN VIENNA, 1920 : TREATMENT WITH COD - LIVER OIL AND PLANT OIL.

BY

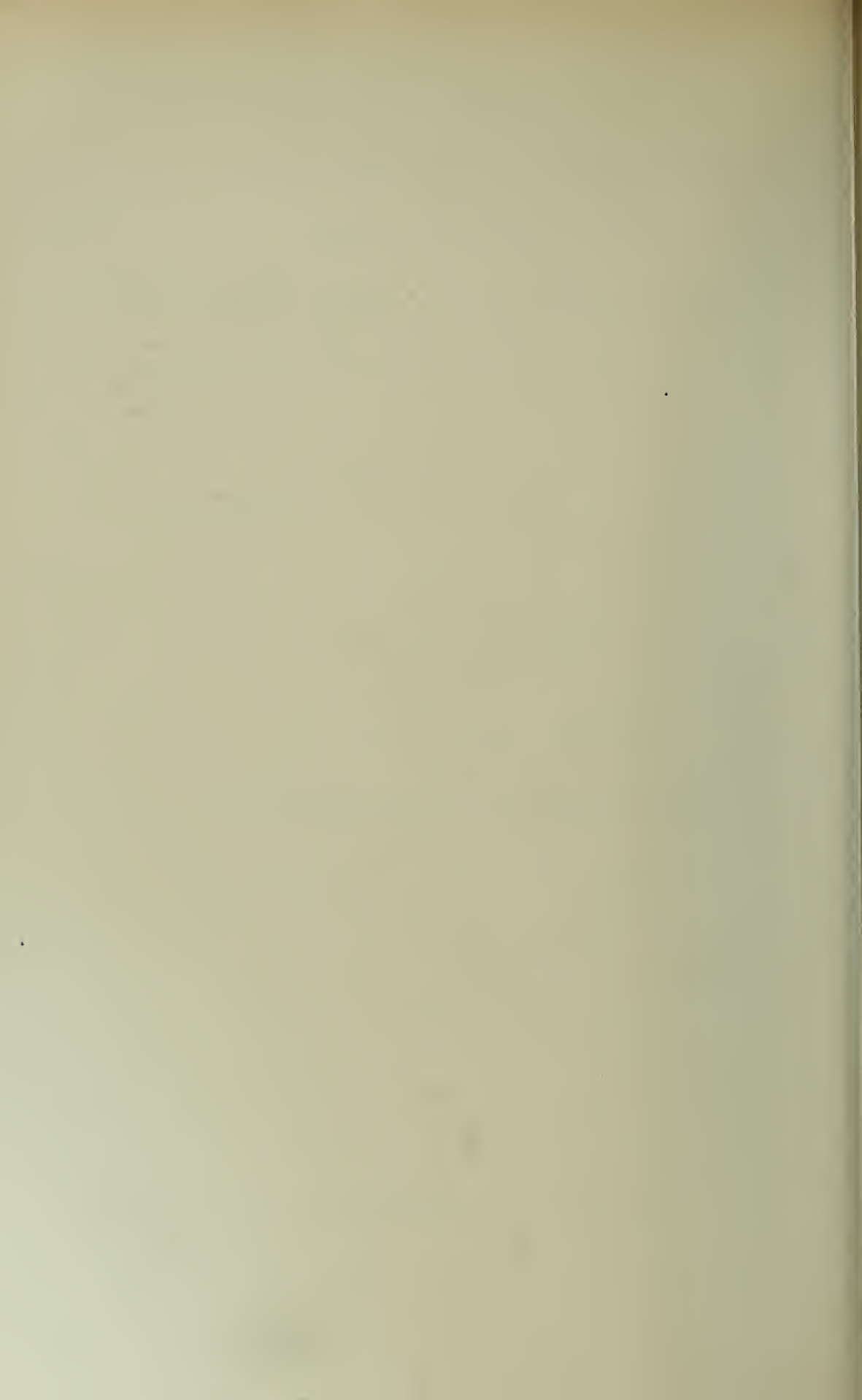
E. MARGARET HUME,
LISTER INSTITUTE;

AND

EDMUND NIRENSTEIN M.D.,
VIENNA.

*A Report to the Accessory Food Factors Committee appointed jointly
by the Lister Institute and the Medical Research Council.*

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COMPARATIVE TREATMENT OF CASES OF
HUNGER-OSTEOMALACIA IN VIENNA,
1920, AS OUT-PATIENTS WITH COD-
LIVER OIL AND PLANT OIL.

AN investigation on 177 out-patient cases of hunger-osteomalacia was made at the central office of the Verband der Krankenkassen Wiens und Niederösterreichs, VI. Königseggasse 10, Vienna. The chief medical officer, Prof. A. Schiff, very kindly offered every possible facility for the research. The investigation took place between February and June, 1920. The central office of the Verband der Krankenkassen is the chief office of a large sick benefit organisation which has a number of branch offices distributed throughout Vienna. The subscribers, when ill, notify the fact to their local branch office and are usually treated there, but in the case of hunger-osteomalacia the method was adopted of sending all patients to be treated at the head office. Presumably this line was taken because the disease was previously unknown and the best method of treatment was not established.

During the year 1919, when the disease was first recognised, 924 new cases presented themselves for treatment between March and December. In the first six months of 1920, 620 new cases came up for treatment, and in addition many old cases were attending regularly. From this mass of material about 200 suitable patients, in whose case the diagnosis was clear and unobscured, were selected for special study in the month of February.

*Previous Treatment and Special Features of the
Krankenkasse Cases.*

The previous treatment at the Krankenkasse had consisted in dosing the patients with a plant oil containing phosphorus (0.01 g. P. per 100 g. oil), a treatment based on the belief that phosphorus has a curative value.* Bottles containing 100–150 c.cm. of the oil were dealt out and patients were supposed to come up weekly for a new bottle, but in practice, living as they did at great distances from the central Krankenkasse, and being severely handicapped in

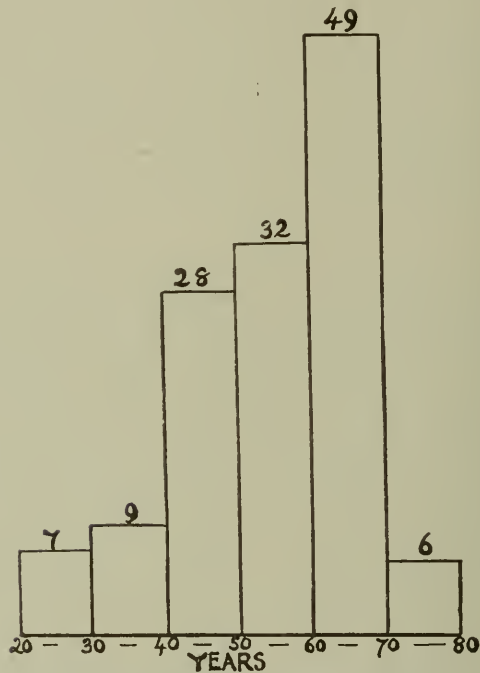


FIG. 1.—Distribution of ages among 131 cases of hunger-osteomalacia.

moving about by their painful disease, they did not come so often. All that can be said as to the dose taken, therefore, is that it was never more than about 20 g. of the oil a day and seldom as much. Many patients stated that they had felt no improvement while taking the oil and many were certainly in a deplorable condition when first seen, although they had had treatment for many months.

* See Schlesinger, 1919 ; Clairmont and Schlesinger, 1914.

Before dealing with the special study there are some points of general interest in connexion with this large group of cases which deserve mention.

Age and Sex of Patients.—The patients were of both sexes, with some preponderance of the female sex; out of 177 cases 44 per cent. were male and 56 per cent. female. It is possible that the female sex is actually more liable to the disease, but it is probable, on the other hand, that the preponderance was due to economic causes—namely, to the greater poverty and consequently worse food of old and single women dependent on their own earnings, for such the cases chiefly were. The patients were all very poor; the largest numbers of cases came from the 10th, 12th, and 16th Bezirks, which are the poorest districts in the city. Many of the cases were very old, and although it is probable that old age does increase the liability to the disease, here also the economic factor cannot be neglected. Of 131 cases whose ages were noted all except 16 were over 40, and more than one-third were between 60 and 70 (see Fig. 1). On the other hand, some persons of all ages from 19 to 77 were seen who were suffering from the disease. At the younger age-limit, under 25, the disease merges into the syndrome of rachitis tarda, of which 10 cases were seen among the 177 selected patients; one or two cases were seen which appeared to be transition cases, and showed the symptoms characteristic of osteomalacia and rachitis tarda at the same time.† Many patients were too old to be in work, and the rest were drawn from a number of different trades, but by far the larger number were unskilled workers.

Diet.—The diet, particularly in the winter, had consisted principally of bread and kraut, the white heart of the autumn cabbage, as opposed to the green kohl, together with root vegetables. Hunger-osteomalacia shows a marked seasonal incidence in winter and spring, corresponding with, it is believed, the season of worse diet. The curve (Fig. 2) was provided by the kindness of Prof. Schiff, and shows the numbers of new cases reporting themselves each month for treatment during 1919 and 1920. The number of new cases reporting sick may be taken as a measure of the prevalence of the disease. Although no patient, as a rule, reported for treatment until he had been ill a month or more, yet patients would not be likely to report for treatment unless they felt themselves getting steadily worse. In each year the apex of the curve comes in the same month—April—but in 1919 fresh cases appeared in considerable numbers through May, June, July till August, when there was a sudden rapid drop. At the time when this drop occurred in 1919 Prof. Schiff reports that he attributed it to the arrival of a large consignment of American bacon in the city. In 1920, however, the great drop in the number of fresh cases came in May, and in June they had fallen to a very low figure indeed, so that this period in 1920 corresponds with August in 1919. In June, 1920, there was no special large influx of American bacon or of any other foodstuff

† For a full account of these symptoms see Edelmann, 1919; Schlesinger, 1919; Chick and Dalyell, 1921.

into Vienna, and it seems probable that the much more rapid diminution in the number of fresh cases in 1920 as the year advanced must be attributed to the exceptionally early spring following on a winter of no severity. The early spring produced salads, green vegetables, and a little meat at a price which the poor could pay six weeks or two months earlier than would normally be the case.

The actual possible bearing of the supply of green vegetables on the ætiology of the disease is discussed

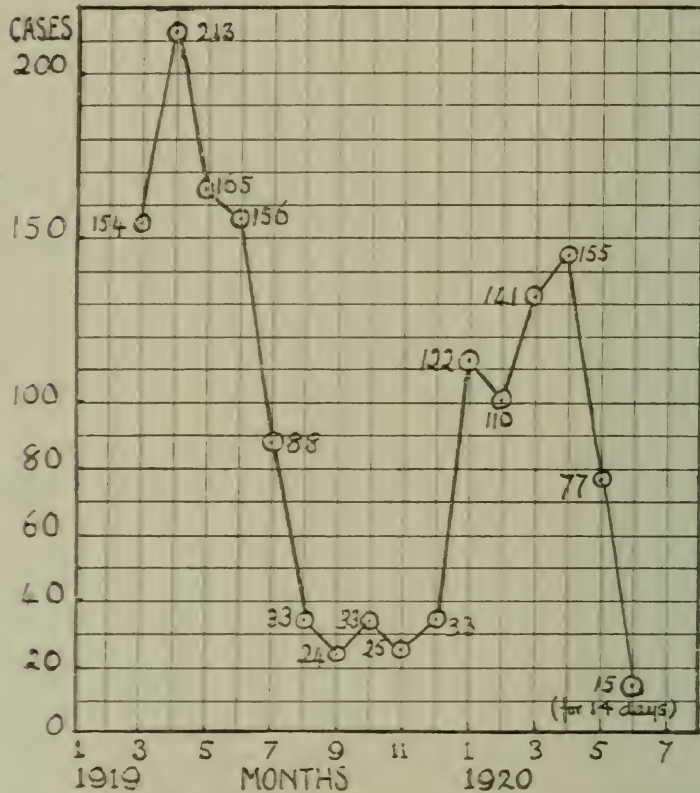


FIG. 2.—Monthly record of new cases reporting sick with hunger-osteomalacia at the Krankenkasse, March, 1919–June, 1920.

elsewhere, but it is as well to make it clear that it is not believed that scurvy played any part. No symptoms of scurvy were ever seen or reported among the patients and the great predominance of white cabbage and roots, even though well cooked, in the diet producing hunger-osteomalacia, makes any incrimination of scurvy almost unthinkable.

*Special Investigation of 177 Cases : Methods
Employed.*

Since one of the writers (E. N.) was a regular member of the Krankenkasse medical staff, it was possible to see the patients in the ordinary course of Krankenkasse business and to distribute treatment through the channels already organised. The treatment given in this investigation was therefore, in many ways, a continuation of the previous treatment and may very fairly be compared with it. It was proposed to treat roughly one-third of the cases with 200 g. weekly of cod-liver oil, ‡ one-third with 100 g. weekly, and one-third with 200 g. weekly of the vegetable oil containing phosphorus, "phosphor oel," which had previously been used in Krankenkasse treatment, and to observe the result. The cod-liver oil used for treatment was obtained through the kindness of several of the relief missions, the Friends' Emergency and War Victims' Relief Committee, the Vienna Emergency Relief Fund, and the International Spitalshilfsaktion, and was the ordinary refined cod-liver oil of commerce. The vegetable oil came from the Vienna municipality central fat control and was unfortunately of unknown origin; a sample has, however, been brought to England, and it is hoped to identify it and test its growth-promoting powers on rats. (See Appendix.)

The method adopted in dealing with the patients was to see them in turn and question them as to the duration of their illness and the mode of onset of symptoms, and any previous treatment which they might have received. The disease was often reported as of long standing (1-36 months). In cases which had been ill for more than a year, a remission was generally admitted in summer, followed by a relapse in winter. Many patients were entirely untreated, others had had treatment with phosphor oil or had been taken into hospital. A few cases treated in hospital had enjoyed remarkable improvement, but just as often this was not the case and none spoke enthusiastically of cure in the past. Almost all cases said that pains had appeared first in the feet and legs, later in the sacrum, and later still in the ribs, but this order of development was not universal.

Three special symptoms were selected as a standard of severity, the gait, the degree of difficulty manifested in

‡ In the whole series of cases only one was found, Marie P., who was unable to tolerate the large amount of oil; on small quantities she made only very slow progress; on large quantities she lost appetite, her digestion became disordered, and the pains due to osteomalacia became more severe.

trying to go upstairs, and the tenderness of the ribs to pressure; for this last symptom it was usually enough to watch the patient's face while exerting pressure on the ribs. According to the severity of each, half, one, two or three crosses were given to each symptom. It is obvious that such a method of standardisation can only give a very rough measure of the severity and progress of the disease, but in a disease which has no more marked objective symptoms than these, it is only possible to adopt some such procedure. Further it was necessary to see a large number of patients in a morning, and no more detailed examination was possible.

To each patient was given a card, with the method of treatment written on it, and he was instructed to obtain weekly a fresh bottle of oil, which would be inscribed on the card by the dispenser. He was further to present himself to the doctor again in about a fortnight. At the next visit, the number of bottles of oil consumed by the patient were noted down by the doctor, but it was never found possible to compel the patients to fetch or send for their bottles at perfectly regular intervals. At each fresh visit to the doctor the patient was put through his paces, and the gait, stair-climbing capacity, and rib pressure pains were charted again with crosses. Most patients showed some improvement after a fortnight, and very marked progress in a month; if they failed to do so, the treatment was usually changed about the fourth or fifth week to cod-liver oil if they were receiving phosphor oil, or to larger doses of cod-liver oil if they were already receiving it. When patients began to improve, the improvement was almost always first manifest in the ribs, and often the ribs were no longer sensitive to pressure at a time when the gait had as yet scarcely shown any improvement. The sacral region and gait then gradually improved, until the walk and stair-climbing appeared normal; but long after all objective symptoms had vanished, the patient still complained of pains about the thighs and feet, particularly in the insteps. In comparing the time required to effect a cure with the three different treatments, it was decided to disregard such purely subjective symptoms; there was no means of standardising them, and although there is no doubt that they were often genuine, sometimes they may not have been, and the desire to continue to obtain a weekly bottle of oil and sick-pay benefit cannot be excluded. All oil, and particularly cod-liver oil, fetched a very high price in Vienna, and a patient almost cured might well wish to sell his weekly bottle. It is not thought that there was danger of such fraud earlier in the disease, for the patients were in great pain and misery; many of them would weep, and their eagerness for the oil was in some cases almost wolfish. In view of the considerations just stated, it was decided that in studying the rate of cure, the course of the disease after disappearance of objective symptoms should be neglected; the term "cure" is therefore used in this study to indicate the time at which all objective symptoms had vanished. It is not to be supposed that treatment ceased at this point.

An attempt was made to weigh patients every time they came up for examination, but it had to be abandoned.

Time did not permit of each patient being stripped, and the change from winter to summer made it impossible for them to come always in the same clothes. The patients were all thin, as is, indeed, the entire poor population, but they were not especially emaciated. Enough information was obtained to show that no very considerable increase of weight accompanied the cure; patients showed perhaps 1 kilo, $1\frac{1}{2}$ or 2 kilos gain over the whole period of two or three months. Of 24 cases who were weighed at the beginning of treatment, and again one to three months later, after a marked improvement in their symptoms had taken place, an average gain of 3.5 kilos is recorded, if a difference of about 3 kilos between the winter and summer clothing is allowed for. Thus it is clear that there was no tremendous regaining of weight, corresponding with the enormous losses some of the patients claimed to have suffered; one case, for example, Juliana B., claimed to have lost 30 kilos, another, Leopoldine G., 17 kilos, and a third, Rudolf N., 20 kilos. Neither did the patients appear much fatter after the cure had set in, although their appearance was much changed; the colour was much better, and the whole mien of the people altered; from being a waiting queue of the most pitiful human wreckage, scarcely able to creep along, ready to burst into tears at a word of questioning, they became a row of brisk individuals, cheerful and extremely grateful.

Analysis of the Results.

In analysing the figures, dealing with the rate of cure in the different treatment groups, a number of factors come into play which tend to destroy the greater accuracy which it was hoped to attain by investigating a large number of cases. In the first place, a certain number of cases have to be rejected altogether, owing to complications with other diseases, irregularity of attendance and similar factors. Among those left, 130 in number, the effect of the interfering factors is to break up the three large groups planned for into a much larger number of smaller groups, groups so small that in many cases they cannot contain sufficient numbers to be representative.

One such factor is the initial severity of the cases; obviously in comparing the length of time necessary for all objective symptoms to vanish, very severe cases and very slight ones could not be taken as comparable. Cases were therefore broken up into four degrees of severity, according to the number of crosses given to the three objective symptoms, as already described; here was wide scope for human error. On working out from the patients' cards the average weekly dose of oil taken, it was found that the oil had been drawn so irregularly that patients fell into six groups, instead of the three planned—i.e., over 150, from 100 to 150, and under 100 g. weekly of cod-liver oil, and the same of phosphor oil. Since each treatment group contained

four different degrees of severity of the cases, 24 groups were revealed instead of the three sanguinely hoped for. When 130 patients have to be divided among 24 groups instead of three, the result loses very much of the reliability that was hoped for from the use of large numbers.

Further possible sources of error were alterations in the patients' diet, either through the change of the season or through free gifts from relief organisations. The latter certainly took place to some extent, but was spasmodic, and did not appear sufficiently important to merit attention. The seasonal alteration in diet might have been a serious source of error, since the investigation was carried on from the end of February to the end of June. About mid-April patients began to report eating spinach twice a week, and by the end of May occasional eggs and a little meat were not infrequent. It is not, however, thought that this factor is an important one in influencing the cures in the present experiment, although it has been suggested as an important factor in determining the number of new cases reporting sick. Patients seldom report sick with osteomalacia until they have had it for several months and are getting steadily worse; to be no longer getting worse, though scarcely perceptibly better, would be enough to deter patients from reporting who had not reported already. The cures with cod-liver oil and large amounts of phosphor oil were, on the other hand, so comparatively rapid that the small added improvement which might be brought about by the consumption of spinach twice a week would be unimportant. Also in many cases cures were well on foot, and great progress had been made before any seasonal alteration in the available food could be detected. It is therefore believed that this factor can be neglected in the analysis of the figures.

The results show clearly that hunger-osteomalacia can be cured by administration of cod-liver oil, even in amounts of only 100 g. weekly, or of phosphor oil in larger quantities, about 200 g. weekly. Clearly the amounts of oil previously administered from the Krankenkasse had not been large enough nor regular enough, though they were probably as large as the smaller amounts of cod-liver oil administered in the experiment. All patients with one possible exception, a very old and refractory case, Max L., improved when treated with 200 g. of cod-liver oil weekly. All patients treated with phosphor oil did not improve in the same way, particularly those who by infrequent visits reduced their weekly average to less than 150 g. When patients after several weeks of treatment showed no or very little improvement, or actually got worse, as was occasionally the case with phosphor oil, the treatment was usually changed from phosphor oil to cod-liver oil or from smaller to larger amounts of cod-liver oil. In every case save one (Max L., phosphor oil 200 changed to cod-liver oil 200), where such a change was made, much better progress took place after the change.

Table I. has been constructed to show (1) the number and percentage of patients who improved on the various treatments ; (2) the number who showed no improvement ; (3) the number who had changed treatment, as just described ; and (4) the result of the changed treatment. In analysing the figures in this way, where some degree of improvement is the criterion taken, cases of all degrees of severity are dealt with together, for although the severity influences the length of time needed to accomplish a cure, yet it does not appear to influence the length of time in which a material degree of improvement sets in. In Table I. the figures dealing with 130 patients are thus analysed, and it is seen

TABLE I.—*Comparative Progress of Patients with Hunger-Osteomalacia Treated with Phosphor (Plant) Oil and Cod-Liver Oil.*

(A) Number of patients showing progress.

(B) Number of patients showing no progress. Treatment unchanged.

(C) Number of patients worse or showing no or very little progress. Treatment changed.

(D)* Number of patients showing greater progress after change.

(E) Number of patients showing no progress after change.

Group.	Treat- ment.	Oil g. weekly.	Total patients.	(A)		(B)	(C)	(D)	(E)
				Total.	%				
I.	Phosphor oil	60-100	2	0	0	1	1	1	0
II.	„	100-150	10	6	60	2	2	2	0
III.	„	150-200	29	21	72	1	7	6	1
IV.	Cod-liver oil	50-100	34	31	91	0	3	3	0
V.	„	100-150	34	32	94	0	2	2	0
VI.	„	150-200	21	21	100	0	0	-	-

that : (A) In Group I., containing two patients who received less than 100 g. phosphor oil weekly, neither (i.e., 0 per cent.) showed improvement, while in Group IV. 34 received the same amount of cod-liver oil, and 31 (i.e., 91 per cent.) showed good improvement ; (B) in Group II. 10 received 100-150 g. of phosphor oil weekly, and 6 (i.e., 60 per cent.) showed progress, while in Group V. 34 received the same amount of cod-liver oil, and 32 (i.e., 94 per cent.) showed good progress ; (C) in Group III. 29 patients received 150-200 g. of phosphor oil weekly, and 21 (i.e., 72 per cent.) showed good progress, while in Group VI. 21 patients received 150-200 g. weekly of cod-liver oil, and 21 (i.e.,

100 per cent.) showed progress. The patients whose treatments were changed are not represented again in the groups for their second course of treatment. For instance, the one patient, Max L., who made no progress after being changed from 200 g. weekly of phosphor oil to 200 g. weekly of cod-liver oil, is mentioned only in the first-named group and not again in the second. The figures show that even 150–200 g. weekly of phosphor oil gives a percentage progress rate (72 per cent.) which falls below the rate (91 per cent.) for quantities of only 50–100 g. weekly of cod-liver oil, pretty clearly indicating that the healing effect must be due to something other than mere fat as fat or phosphorus. The percentage progress rates for the three cod-liver oil groups lie very close together—i.e., 91 per cent., 94 per cent., and 100 per cent.—showing that in most cases even 50–100 g. weekly of cod-liver oil is sufficient for cure.

TABLE II.—*Comparative Rates of Cure of Cases of Moderate Severity (+), whose Treatment Remained Unchanged Throughout.*

Treatment.	Total No. of patients.	No. of weeks in which cure was completed (A), or after which treatment ceased, though cure still incomplete (B).													
		2	3	4	5	6	7	8	9	10	11	12	13	14	
Phosphor oil, 100-150	2 { (A) (B)	0 2	- -	- 1	- -	- -	- 1	- -	- -	- -	- -	- -	- -	- -	
Phosphor oil, 150-200	12 { (A) (B)	9 3	1 1	- 2	2 -	1 -	3 -	1 -	- -	1 -	- -	- -	- -	- -	
Cod-liver oil, 50-100	22 { (A) (B)	18 4	1 1	- -	- -	3 2	5 -	1 1	2 -	- -	2 -	- -	2 -	1 -	
Cod-liver oil, 100-150	17 { (A) (B)	17 0	- -	1 -	2 -	3 -	2 -	3 -	3 -	- -	2 -	- -	- -	1 -	
Cod-liver oil, 150-200	8 { (A) (B)	6 2	- -	1 1	1 -	- -	2 -	- -	- -	- -	- 1	1 -	- -	- -	

An attempt has been made in Table II. to compare the rate of cure on different treatments of patients whose treatment remained the same throughout. For this the patients needed to be as comparable as possible, and the group of patients of moderate severity (+) has therefore been chosen, because it contains the largest numbers. In each treatment group a certain number of cures were unfinished—that is to say, that although good progress was being made, all objective symptoms had not disappeared on the last occasion on which the patient was seen. These unfinished cures are represented in the table because they cannot fairly be entirely neglected, but they are left out of consideration because they are inconclusive. The table

shows that in the five treatment groups represented the dates of "cure" ("cure" being used in the special sense already described p. 5) distribute themselves pretty equally from the second to the tenth week, with a few as late as the fourteenth week. There is certainly no great superiority in rate of cure in any one group over any other group. Such a table suggests that as soon as sufficient of the curative substance is supplied rate of cure is not appreciably hastened by more intensive treatment; probably the maximum rate of repair has been attained, but it is also possible that repair at an even more rapid rate may be only prevented by an insufficient supply of something else, the lack of which acts as a limiting factor.

The Possible Value of Phosphorus.

The work of Schabad (1909, 1910) on calcium retention in the cure of rickets by treatment with oils suggests that an insufficient supply of phosphorus may have been such a limiting factor. He found that cod-liver oil had great power in increasing calcium retention in rickets and that this power is further heightened on addition of phosphorus to the cod-liver oil. If, then, there is a parallel between hunger-osteomalacia and rickets, it is possible that addition of phosphorus to cod-liver oil might also hasten the cure of osteomalacia. It is unfortunate that in our experiments the vegetable oil had an addition of phosphorus while the cod-liver oil had not; had the cod-liver oil also contained phosphorus it is possible that its superiority over the vegetable oil might have been even more marked. Schabad also found that certain vegetable oils had power to increase calcium retention in rickets, but in the case of sesamum oil this was not so, and addition of phosphorus to the inactive oil failed to awake any activity in it. His work, therefore, makes it clear that the coöperation of another factor in the oil is required and that the absence of such a factor may entirely limit any curative power of the phosphorus.

From the present experiments it would therefore seem that in the treatment of hunger-osteomalacia a dose of about 100 g. weekly of cod-liver oil is the most economical form of therapy to commence with in all cases, increasing the amount later only in those refractory cases which make little or no progress upon this dose. From the present experiments no conclusion can be drawn as to the possible coöperative value of phosphorus. Reference to one particular case is made in more detail; it is a particularly striking one but is also characteristic of a large

number in which the result of treatment with cod-liver oil stands out in contrast with that of the former routine Krankenkasse treatment with phosphor oil.

The patient, Wilhelmine O., gave a history of having been ill for 30 months; for nine months, March, 1919, to January, 1920, she had been receiving the routine Krankenkasse treatment with phosphor oil, which left her condition unchanged. When first seen at the beginning of March, 1920, she was unable to dress or undress herself, and could not even turn her head without pain. She showed a rapid improvement on cod-liver oil, 125 g. weekly, and by the first week in June—i.e., after three months of treatment—described herself as “Vollständig gut,” save for a slight pain in the lumbar region. Thus all objective symptoms and almost all subjective ones also, of an illness lasting 30 months, were dissipated in three months by rather more than one tablespoonful of cod-liver oil daily. This patient had been known to one of the writers (E. N.) for the whole 12 months during which she had attended at the Krankenkasse, and the unaltered condition during the nine months of phosphor oil treatment, followed by the cure on three months cod-liver oil, was most impressive.

When patients were considered to be cured the treatment was discontinued, and as far as it was possible to judge in the time, relapse did not tend to take place. Treatment was in no case stopped till May or June and the experiment was terminated at the end of June. Patients when sent away were instructed to come back at once if they felt worse, and of over 70 patients so discharged only three or four had returned announcing a relapse; in these cases the relapse only consisted in a reported return of subjective symptoms which may or may not have been truly stated. It seemed most probable that the patients would remain in health through the summer but would relapse again towards the end of the year unless treatment were resumed or the food obtainable in the winter became better.

Effect of Treatment on Cases of Rachitis Tarda.

Of the 10 cases of rachitis tarda encountered, 4 cases (2 boys and 2 girls) were chosen out for in-patient treatment and will be described in a later communication. The other 6 were all boys, one 16, one 17, one 18, two 19, and one 20 years of age. Three dosed with 200 g. of cod-liver oil weekly, and one dosed with 100 g., made excellent steady though slow progress; two dosed with 200 g. weekly of phosphor oil, but very irregularly taken, made little or no progress.

*Bearing of the Results on the Aetiology of the
Disease.*

As far as the present mass experiment throws light on the aetiology of hunger-osteomalacia, it seems to support the hypothesis that the disease is due to a deficiency of the fat-soluble accessory factor. The disease was cured, and dramatically cured, by an addition of fat only to the diet. It is true that the plant oil contained phosphorus, but the cod-liver oil had no such addition, though it may itself have contained phosphorus to some extent. It has already been suggested that the absence of phosphorus in the cod-liver oil may have acted to some extent as a limiting factor, but there is no evidence that the rate of cure bore any relation to the amount of phosphorus administered. There is evidence, on the other hand, that the cod-liver oil cured in quantities in which the plant oil did not, and that bad or obstinate cases yielded to cod-liver oil when they would not do so to plant oil. This evidence seems good enough to exclude fat as fat and to point to some other property, possessed in greater measure by cod-liver oil, as the curative agent. One such property cod-liver oil is known to possess in its rich content of the fat-soluble A accessory factor.

The difference between the winter and summer diet of the population and the corresponding seasonal alteration in the severity and prevalence of the disease yields another interesting piece of evidence, connecting the disease with a deficiency of the fat-soluble A factor. The winter diet of the poorest of the Vienna population is chiefly bread, "Kraut," and root vegetables, while the summer diet is principally enriched by the addition of green vegetables and salads. The kraut used in winter is the white hearts of cabbages, which are stripped of their green leaves in the autumn and are then stored in the same way as root vegetables. Unpublished experiments at the Lister Institute, on guinea-pigs, have shown that while green cabbage is growth-promoting even in small amounts, white cabbage in much larger amounts is not so; for instance, 5 g. of raw green cabbage, when added to a diet deficient in the fat-soluble A factor produces good, though not fully normal, growth in guinea-pigs, while 15 g. of white cabbage added to the same diet produces no growth at all. A similar observation for other etiolated shoots has been made by Drummond and Coward (verbal communication), using young rats. The white leaves are, however, not deficient in the anti-scorbutic

factor (Delf, unpublished experiments). The bread and root vegetables which make up the remainder of the diet of the poor have been shown again and again, with the exception of carrots, by McCollum and his co-workers to be deficient in the fat-soluble accessory factor.

More evidence is needed in order to clear up completely the question of the ætiology of this disease, and it is possible that in the deplorable event of the same conditions prevailing in Vienna during another winter such evidence may be obtained. The ætiology of hunger-osteomalacia has not only the interest that it is the ætiology of a new disease, but it has also great importance from its relation to rickets through rachitis tarda and from the light which a knowledge of its ætiology may throw upon the ætiology of rickets. Whether it is the same disease as the already well-known osteomalacia of pregnancy appears very uncertain. Among the Krankenkasse cases only one, Marie L., was encountered that had any claim to be diagnosed as osteomalacia of pregnancy. This case was that of a woman beyond the child-bearing age, who, however, gave a history of osteomalacia during pregnancy going back to 14 years before; several of her children had suffered from severe rickets. If pregnancy-osteomalacia and hunger-osteomalacia are the same disease it is hard to understand why the former has not greatly increased in Vienna, but it has not done so. It cannot be that such cases were wholly overlooked; it may be that economic conditions do not on the whole press so severely on young women of the child-bearing age, who would in most cases have a husband to support them, but unless some such factor is operative it can scarcely be that hunger- and pregnancy-osteomalacia have the same ætiology.

Summary.

1. 177 cases of hunger-osteomalacia were treated as out-patients in Vienna. Of these, only 130 entered into the final analysis of the experiment.

2. About one-third of the number was treated with a plant oil (subsequently identified as belonging to the rape oil group) containing phosphorus, the other two-thirds with cod-liver oil.

3. Most of the patients made progress, but some of the patients on plant oil had to be transferred to cod-liver oil, and some of those on cod-liver oil had to have the dose increased before good progress could be made.

4. The results fall into a series; doses of about 100 c.cm., 150 c.cm., and 200 c.cm. of each of the two kinds of oil were given. The smallest doses of plant oil gave the worst result and the largest doses of cod-liver oil the best; the smallest dose of cod-liver oil was better than the largest dose of plant oil.

5. Once a good improvement was set on foot it could not be detected that the rate of progress on the different doses varied much.

6. It is provisionally concluded that cure of the disease was due to addition of vitamin A rather than of fat as fat to the diet.

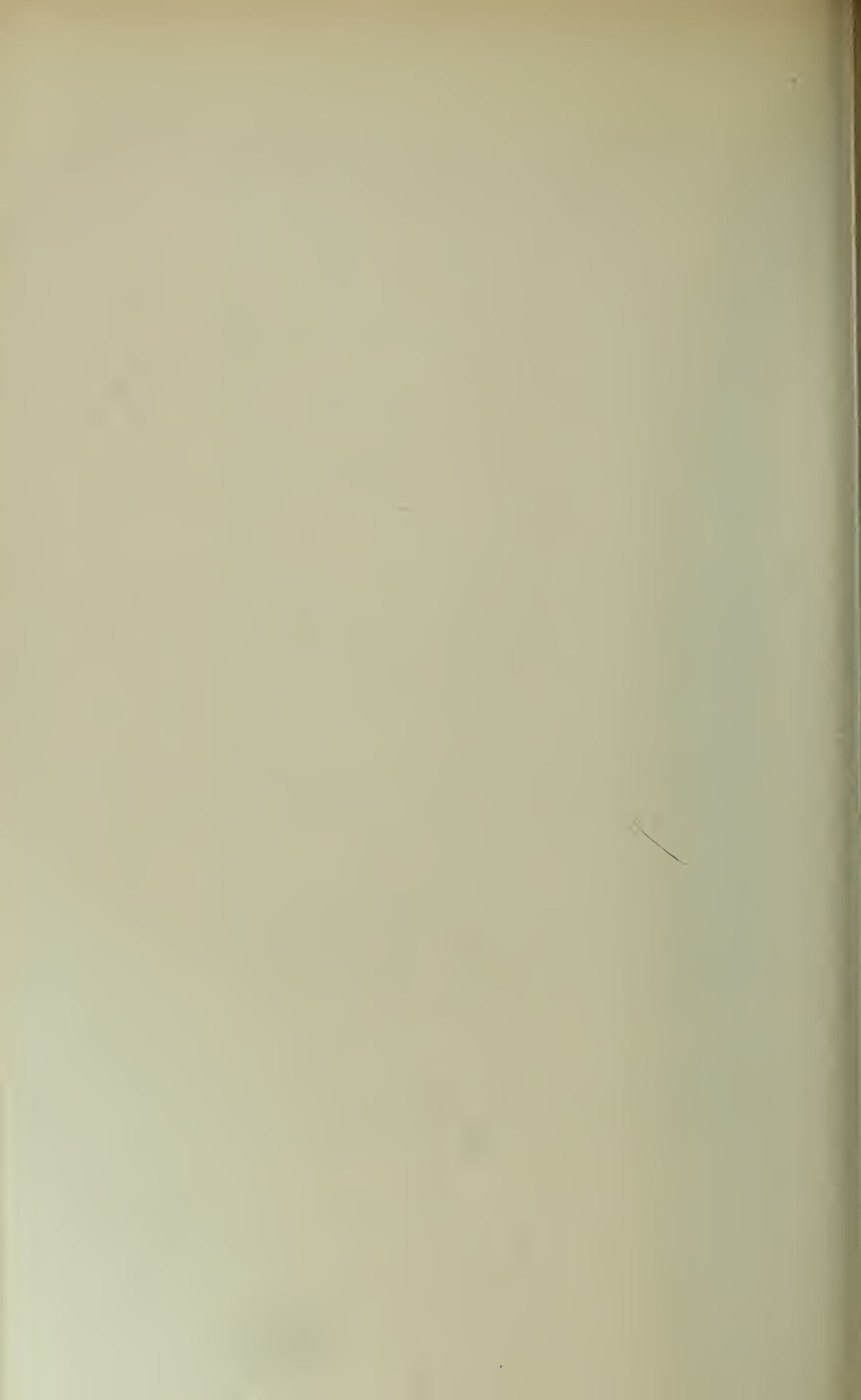
7. The relation of hunger-osteomalacia to the osteomalacia of pregnancy is regarded as quite uncertain.

The expenses of this research were defrayed by the Medical Research Council.

Appendix.

A sample of the phosphor oil used in the above test was submitted to Dr. J. C. Drummond at University College, London, who reported that he had little hesitation in placing it in the rape oil group; in fact, he adds: "It is probably true rape oil itself. It appears to be fairly crude, since there is a considerable deposit of recognisable plant debris." The oil was also tested by Dr. Drummond on rats for its value in vitamin A. It appeared to be almost, if not wholly, incapable of restoring growth in rats which had declined on a diet deficient in vitamin A, in the quantities of it able to be consumed by rats; such quantities are, however, necessarily small. The authors are much indebted to Dr. Drummond for undertaking this examination for them.

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N^o 10

EXPERIMENTAL RICKETS IN RATS.

BY

V. KORENCHEVSKY, M.D.

(From the Department of Experimental Pathology, Lister Institute.*)

Historical.

THE earliest attempts to produce rickets experimentally in animals were by administering food deficient in calcium (E. Voit, Baginsky, Aron and Sebaauer, Götting, Miva and Stoelzner, Dibbelt and others), or in phosphates (Heubner). The view that rickets is an infectious disease receives some support from the experiments of Morpurgo, who isolated a diplococcus from spontaneous outbreaks in rats, and found that rickets ensued after he injected cultures into the animals, provided the inoculations were made at a sufficiently early age. Confinement and limitation of muscular exercise as a possible etiological factor in rickets has been studied by Findlay, Paton, and Watson, and is considered of importance by them.

The dietetic theory has recently been revived by Mellanby, whose investigations indicate that those constituents of a diet which are particularly rich in fat-soluble vitamin A possess a profound importance in connexion with the occurrence of rickets in puppies. Mellanby's conclusion is to some extent supported by the experiments of McCollum, Simmons and Parsons, Shipley and Park; but the American observers, who made their observations upon rats, found that changes more closely resembling rickets occurred in these animals when their diet, in addition to being deficient in vitamin A, was also poor in calcium salts or phosphates. Hess and McCann and Pappenheimer, who also experimented on rats, found that a diet deficient in vitamin A did not produce rickets but lack of active osteogenesis.

Lastly, different abnormalities in the function of endocrine glands have been connected with the etiology of rickets (Erdheim, Stoelzner, and others), important experiments having been conducted on this question by Erdheim.

The theories deduced from all these investigations have numerous adherents and numerous opponents.

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present in children who are emaciated and do not grow, although obvious rachitic changes in the bones begin to develop only from the moment when the organism starts growing.

Looser's investigation on "rachitis tarda" in patients whose growth was retarded and nourishment frequently poor is an important contribution to the question of rickets viewed from the above standpoint.

This excellent investigation has shown that in such patients, as well as the usual manifestations of rickets, atrophy of the bones occurs. The occurrence of osteoporosis is, however, not infrequently referred to as an argument against the diagnosis of rickets (Schmorl). The amount of osteoid tissue and the development of subchondral spongiosa appeared to be small in the cases investigated histologically by Looser. The development of periosteal osteophytes was not always observed, and, when observed, was moderate in amount. On the other hand, Ziegler regards osteoporosis as frequently accompanying rickets.

Returning to my own experiments, and taking into consideration that animals kept on diets —A and —A —Ca were in most cases in a state of retarded or inhibited growth and of more or less profoundly disturbed nutrition, one would on *a priori* grounds expect rachitis, if it is developed, to appear more in the form described by Looser.

In the diagnosis of rickets in rats I have been guided by Erdheim's excellent work on spontaneous rickets in these animals.

Whether obvious bony deformities, bending, swelling of epiphyseal junctions, spontaneous fractures, etc., were present or not, I have considered the following conditions to be essential for the diagnosis of this disease:

1. Diminution of calcium content in bones.
2. Presence of osteoid tissue in amounts distinctly exceeding the normal.
3. Enlargement and disorganization of the zone of proliferating cartilage.
4. Absence or defect of deposition of lime salts in the zone of provisional calcification.
5. Presence of periosteal osteoid tissue.
6. Absence or marked deficiency of calcareous deposition in callus after spontaneous fractures of bones.

The presence of osteoporosis in the conditions named above, as observed in a certain number of our animals, does not prevent the diagnosis of rachitis.

*Effect of Feeding on the Basal Diet but without
Calcium in the Salt Mixture.*

The amount of calcium in the basal ration was 0.04 per cent., so that, assuming the animals consumed the whole ration, the maximum amount of calcium ingested daily was approximately 8 mg. Two types of experiments were conducted upon 13 animals in all. In the first, consisting of 9 rats, the deficient diet commenced when they were about 30 to 50 grams in weight. The experimental animals remained behind the control ones in

weight, but not to such a degree as on -A diet. The rats also manifested nervous excitability and shyness.

The changes in the skeleton agree in general with those described by previous authors, and are characterized by osteoporosis with narrow layers of osteoid tissue. The zone of proliferating cartilage is but slightly changed and nearly always well calcified. Osteoporosis observed in such cases finds explanation in the increased number of osteoclasts. Spontaneous fractures are very rarely observed. The amount of calcium in the dried bones, however, was about 43 per cent. below normal, and the amount of H_2O was 23 per cent. above normal. The picture that resulted cannot be defined as rickets.

The results were quite different when the young rats kept on N -Ca diet originated from a mother who herself had been kept on this diet during the lactation period. Hitherto I have conducted only one experiment on 4 young rats. These all showed the same changes. The bony deformities were pronounced, and to the naked eye closely resembled those of human rickets. The amount of calcium decreased on the average to 64 per cent. below the normal and the amount of H_2O rose to 44-55 per cent. above the normal—that is, the calcium in the skeleton was greatly diminished. Microscopically, the enlargement of the zone of proliferating cartilage was more obvious—in one case to the extent of 20-27 layers of hypertrophied cells. This zone either contained no calcium at all or it appeared in the form of separate patches or bands, usually near the perichondrium. The ingrowth of vessels into the cartilage, when observed, was not deep, and the line of the costochondral junction was not markedly altered, and bent towards the bone marrow.

The spongiosa consisted of numerous trabeculae which formed a network and were thin in some rats and thicker in others. The layers of osteoid tissue were of different thickness, thin ones predominating. The cortical bone was in a state of osteoporosis. The periosteum was in places hypertrophied. The osteoblasts were in many places well defined and numerous, sometimes being arranged in several rows. The number of osteoclasts was also very great. Similar pictures in pups have been described by Dibbelt, and by Schmorl.

When there is much new osseous formation one notices that calcification has occurred over considerable areas also to a different degree, notwithstanding the fact that the calcium content of the bones was 64 per cent. below normal. These facts produce the impression that the incomplete calcification of the bone is due to calcium starvation.

In human rickets in remission calcium is as easily deposited as normally in the osteoid tissue (Schmorl), but during the process of the disease this is prevented by some cause. This difference I consider to be *essential and of great importance*.

Effect of Feeding upon the Basal Diet with the Complete Salt Mixture in which the Butter and Cod-liver Oil were replaced by an Equivalent Amount of Cotton-seed Oil.

The amount of vitamin A in this diet must have been very small.

The experiments arrange themselves naturally into three groups. In the first, the diet commenced about the age of 30 to 50 days; in the second, the mother received the deficient diet during lactation; and in the third, the mother and the father were subjected to the same dietary deficiencies before conception. In both the latter groups the same deficient diet was fed to the offspring as soon as they were capable of feeding themselves. Twenty-three animals were placed on this diet at the age of 30 to 50 days. In most cachexia developed earlier or later, and the rats became very susceptible to casual infections.

The changes occurring in the skeleton of many such cachectic rats can be characterized briefly as osteoporosis with a lack of signs of active osteogenesis and frequently increased activity of osteoclasts. The calcium content of such bones is not greatly decreased, being about 14 per cent. less than normal.

In those rats in which growth occurred, and in which the bones were examined before cachexia ensued, less osteoporosis, more osteoid tissue, and a small increase of the proliferating cartilage, were observed, and sometimes defective deposition of calcium in the zone of provisional calcification. Corresponding to this appearance the calcium content of the bones was 21 per cent. below normal. In a few cases the appearance was suggestive of slight rickets.

In one experiment a rat, after giving birth to six young, was placed on -A diet during the whole period of lactation of the young rats. The young rats, after having been separated from the mother at the expiration of a month, remained on -A diet till the time when they were killed. The chemical composition of the bones of these rats showed further deterioration, the calcium being 36 to 44 per cent. below normal, and the water 17 to 50 per cent. above normal. In accordance with this low calcium content, numerous spontaneous fractures were observed in the ribs of the young rats. However, microscopical examination revealed the picture of osteoporosis typical for -A, and in four cases osteoid tissue was observed. The thickness of the osteoid was equal to, or even somewhat exceeded, that of the calcified bone in three cases.

As is known, it is a rule that rats kept on -A diet have no offspring, and therefore the males are usually left together with the females. As an exception, however, they do bear young. This was recently observed amongst my rats in one case and amongst the rats of Dr. Zilva at the Lister Institute in three cases. I availed myself of this material in order to determine whether the feeding of parents on -A diet during the periods of conception, pregnancy, and lactation has any deteriorating effect on the condition of the skeleton in their offspring. After separation from the mother the offspring continued to be kept on -A diet. Of these, numbering 18 altogether, I have examined up to the present 12, the offspring of three mothers; 12 were examined histologically, and the skeleton of 7 of these was examined chemically. The investigation was conducted sixty-four to seventy-nine days after birth. In 7 of these 12 rats great proliferation of the cartilage cells and growing-in of blood vessels, and a picture more or less suggestive of rickets, was observed, and in 2 cases the histology was typical of rickets. The average calcium content in the whole series was 25 per

cent. below normal and the water 30 per cent. above normal.

To sum up: As the result of feeding the mother during lactation both on the N - Ca diet (4 rats) and on the -A diet (6 rats) the composition of the skeleton in the offspring deteriorated sharply, but changes closely resembling rickets were obtained in an appreciable number of cases only when feeding on -A diet had been started during conception and pregnancy. In other words, if these experiments were confirmed repeatedly on a large number of animals, they would point to the significance of the condition of the parents, especially the mother, in the origin of the profound changes in the skeleton, in certain particular cases on -A diet even amounting to typical rickets. The supposition frequently advanced regarding the importance of normal nutrition of the parents at the period of conception, and of the mother during pregnancy and lactation, find experimental confirmation in our investigations as well. From this point of view the commencement of rachitic changes in the child could be sought for, not only in the conditions of its own life but also in the state of health—that is, nutrition—of its mother, and maybe of its father as well.

Vitamin A is apparently closely related to the metabolism of calcium in the organism, and particularly in the bone. The experiments of Schlabad, Mellanby, Shipley, Park, McCollum, Simmonds, and Parsons, and those related above, support this view. Its action may not inappropriately be compared with that of an antioceptor, provided the analogy be not pressed too far.

Effect of Feeding upon the Basal Diet and Salt Mixture from which both Calcium and Vitamin A have been largely Removed.

This was investigated upon 27 rats. With the exception of eight, the deficient dietary commenced when the animals were three weeks to one month old. In eight cases (two families) deficient diet was given to the mother during lactation also.

The results confirm those of McCollum and his collaborators upon this point. Combined deficiency in calcium and vitamin A in the food has, in my experience, proved the most certain method to produce rickets experimentally in rats.

Rats kept on this diet manifested deformities of the skeleton which are both macroscopically and microscopically typical of rickets, provided that feeding commenced at a sufficiently early age. The amount of calcium in the bones decreased to about 46 per cent. below normal, and the amount of water increased to about 32 to 46 per cent. above normal, whilst the majority presented all the pathologic manifestations of rickets in a pronounced form; in some rats, kept on -A - Ca diet, the amount of osteoid tissue, although abnormally increased, was comparatively small. In these cases osteoporosis was especially marked, and calcium was not infrequently deposited in the zone of provisional calcification.

In animals fed on the basal diet without the saline mixture the resulting changes did not differ from those produced by deprivation of calcium alone.

Effect of Exclusion of Phosphates from the Salt Mixture.

Deprivation from phosphates in conjunction with fat-soluble deficiency was found by Shipley and Park and McCollum and Simmonds to be followed by changes indistinguishable from those of rickets. In three experiments, comprising 14 rats kept upon N and -A diets respectively, I have removed the phosphates from the salt mixture, at the same time compensating for the loss of sodium and potassium and calcium. As compared with the corresponding control animals, no special changes were observed in the bones. This apparent discrepancy is no doubt attributable to difference in phosphorus content in the basal diets used.

Effect of Castration.

Forty-five rats were castrated, seven of which were deprived both of sexual glands and spleen. When subsequently placed upon the four diets (N, N - Ca, - A, and - A - Ca) no marked influence on the chemical and histological composition of the skeleton as compared with controls was observed. My experiments did not reveal that beneficial effect which is sometimes clinically observed in man in developed osteomalacia. In some of the castrated rats even greater changes were observed in the skeleton, as compared with the corresponding control animals—for example, on -A diet. Therefore one can only state that castration performed before subjecting the animals to the special diet *did not prevent the development of rachitic changes* in the skeleton.

Conclusions.

1. The results obtained by me agree in general with the results of the experiments of Mellanby, McCollum, Simmonds, Parsons, Shipley, and Park. /
2. Confinement in small cages does not evoke rickets in rats.
3. The introduction of live cultures of *B. perfringens*, *B. sporogenes*, and *B. bifermentans* with the food, and of *B. sporogenes* and *B. bifermentans* subcutaneously, produced no visible effect on the development of rickets in rats.
4. The deficiency of the diet in calcium alone can produce changes in the skeleton of rats which present some resemblances with rickets, especially when the young rats have originated from a mother kept on the same diet during lactation.
5. Usually deficiency of food in vitamin A produces in rats impoverishment of the bones in calcium, enrichment in water, and osteoporosis with deficient osteogenesis, and in some cases a picture resembling slight rickets. Changes in the skeleton more similar to rickets, and in some cases typical of rickets, were observed in young rats on -A diet, provided their parents had been fed on -A diet during conception, pregnancy, and lactation.
6. Vitamin A has a relation to the metabolism of calcium in the organism and particularly in the bones, and therefore to the development of rickets.

7. My few experiments on the feeding of parents on food deficient in vitamin A or calcium during conception, pregnancy, and lactation suggest that this may start disturbances of metabolism in the child which, if the deficient dietary be continued after birth, result in serious disorders of the skeleton.

8. The changes typical of rickets occur most readily and most frequently in rats kept on a diet deficient both in vitamin A and calcium.

9. Castration performed before the commencement of feeding has no marked influence on the chemical and histological changes in the skeleton of rats fed on a normal diet, on diets deficient in calcium or vitamin A, or deficient in both.

In conclusion, it is my pleasant duty to record my gratitude to the Medical Research Council for affording me a grant which has enabled me to carry out this investigation, and to the Lister Institute for the hospitality of its laboratories. I am also deeply grateful to Professor C. J. Martin for his continuous sympathy and support. To Dr. R. Robison I express my thanks for advice connected with the chemical investigations; to Miss E. Luce for assistance in operations; to Miss S. Rutherford for help in the work of feeding the animals.

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XXXIII. THE MINIMUM NITROGEN EXPENDITURE OF MAN AND THE BIOLOGICAL VALUE OF VARIOUS PROTEINS FOR HUMAN NUTRITION.

BY CHARLES JAMES MARTIN AND ROBERT ROBISON.

From the Lister Institute, London.

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HISTORICAL.

UNTIL comparatively recently, the search for the minimum protein requirements of the human body has been made on the assumption that protein is an entity, little regard being paid to whether the proteins were derived from meat, milk, cereals, etc.

The more important of the numerous investigations undertaken with this object are those of Hirschfeld [1887], Kumagawa [1889], Klemperer [1889], Peschel [1891], Lapique and Marrette [1894], Sivén [1900] and Albu [1901]¹.

In all of them the protein fed was derived from more than one source and often from several. The observations, which vary in precision, were made under conditions which were not uniform, particularly in regard to the total calories taken. Nevertheless, each experimenter succeeded in establishing that nitrogenous equilibrium could be maintained over short periods with one-third to two-thirds the standard laid down by Voit of 118 g. protein, equal to 0.39 g. N per kilo.

The minimum arrived at by the above experimenters varied between 0.08 g. and 0.18 g. N per kilo, most of the results being round about 0.1 g. The lowest value is that of Sivén, who considers that he ultimately attained nitrogenous equilibrium on a mixed diet containing 0.08 g. N per kilo, only 0.03 g. of which he regards as true protein, but as the only evidence that this small amount was sufficient is a positive balance of .04 g. N on the last day of a four days experiment, decided negative balances occurring on the first three days, this conclusion would appear questionable. In another series in which the nitrogen intake was 37 % higher the evidence of nitrogenous equilibrium is satisfactory.

¹ The earlier observations have been collected by Atwater and Langworthy in their "Digest of Metabolism Experiments," *Bulletin* 45, U.S. Dept. of Agriculture, 1898. An excellent review of the work previous to his paper is given by Sivén. Most of the literature on the subject to date is referred to in Mendel's "Theorien des Eiweissstoffwechsels," *Ergebnisse der Physiologie*, 11 Jahrgang, 1911; Caspari's article "Eiweissstoffwechsel" in Oppenheimer's *Handbuch der Biochemie*, 1911, and Cathcart's *Physiology of Protein Metabolism* (1921). All of these contain good bibliographies.

The investigations of Neumann [1902] and Chittenden [1904] were undertaken with a somewhat different object, namely, to ascertain whether health and activity could be maintained over prolonged periods on a mixed diet of low content in protein. Neumann made three experiments on himself, each lasting four to ten months. The total calories of the diet amounted to 30-40 per kilo. Chittenden's observations were made upon 26 individuals and the duration varied between six and nine months in different cases. The total calories varied between 35 and 45 per kilo body weight. Nitrogenous equilibrium was obtained by Neumann on an intake of 0.15 g. N per kilo and in Chittenden's experiments with 0.1 g. to 0.17 g. per kilo in the different individuals. There is no reason to suppose that these figures represent minima. The conclusion drawn is that a nitrogen intake of one- to two-thirds of the amount laid down by Voit is sufficient to maintain health and efficiency over the periods during which the observations endured.

During the last decade of the 19th century, physiologists were becoming increasingly alive to the possible significance of differences discovered in the elementary composition and chemical properties of the proteins, and the uniform value hitherto attributed to them in nutrition was coming under suspicion. Rubner [1897] appears to have been the first to formulate the view that proteins had different biological values. He used this conception to interpret some early experiments of his on the utilisation of various food-stuffs [1879] in which he had observed that less nitrogen was excreted in the urine on potato diets than on bread diets although the adverse N-balance was smaller on the former. In the same article Rubner expressed the opinion that the old search for a protein minimum must be fruitless since there will be, not one, but many minima according to the nature of the foodstuff used. He does not appear to have attributed such variation to any difference in chemical constitution although doubtless this possibility was in his mind. At this date the chemical constitution of the proteins was obscure although a number of amino acids had been isolated from the decomposition products of proteins and differences in the amounts of these had been observed. A little later Kossel and Kutscher [1900] determined the histidine, arginine, lysine and ammonia derived from the hydrolysis of a number of proteins and Kossel [1901] as a result of his own and others' work came to the conclusion that the habit of regarding protein as a physiological unit was unsound and that, since proteins possess different chemical compositions, they will also have different values for the organism.

About the same time discoveries were being made in another direction. The researches of Cohnheim [1901, 1906], Kutscher and Seemann [1901], Abderhalden and his co-workers and others, proved that a much more exhaustive break up of the protein molecule than had previously been supposed takes place in the small intestine prior to absorption, and that what the body really receives is a mixture of amino acids and simple polypeptides. Loewi [1902], and later, Abderhalden and Rona [1904] showed by means of feeding

experiments with previously digested proteins that the abiuret products of such digestion were capable of maintaining animals in nitrogen equilibrium. Abderhalden believed at first that such amino acids were at once utilised for building up of blood and tissue proteins and in conjunction with Samuely [1905] attempted to ascertain whether the composition of the body proteins varied with the character of food proteins. A horse was fed for three days on gliadin containing 36.5 % of glutamic acid, but no increase in the very low content of this amino acid in the serum proteins could be detected. From these results Abderhalden and Rona [1906] drew the conclusion that in the renewal of body proteins a proportion of the amino acids arising from the food will be left over unless the body is capable of synthesising one amino acid from another. Since this proportion will depend on the relative composition of the food and body proteins the protein minimum must also be variable.

These discoveries provided a theoretical basis for Rubner's empirical conclusions and a stimulus for the further investigation of the subject.

Meanwhile the rôle played by protein in satisfying the energy requirements of the body, and the effect on the protein minimum of insufficient as against abundant provision for these needs from non-protein sources, was becoming more clearly realised.

The discovery of the variable composition of proteins and of the fact that certain of them are almost entirely deficient in one or more of the amino acids was followed by interesting researches to determine to what extent the animal body could, by the practice of economy or synthesis, dispense with the missing complexes. The deficiency of gelatin in tyrosine was ascertained early and the absence from it of cystine and tryptophan was discovered when these amino acids became known as protein constituents. The inability of gelatin to preserve the body in nitrogenous equilibrium has been shown by many investigations, but as this aspect of the subject has recently been dealt with by one of us in this journal [Robison, 1922, 1] it is unnecessary to review it here.

The discovery of tryptophan by Hopkins and Cole [1901] and of the deficiency of this amino acid in zein by Osborne and Harris [1903] was followed by an experimental enquiry by Willcock and Hopkins [1907] to ascertain whether zein would serve as an exclusive source of nitrogen for mice and if not whether the addition of tryptophan would enhance the nutritive value of this protein. Zein alone failed to maintain the animals but this was achieved by supplementing with tryptophan.

Following this pioneer work Osborne and Mendel [1911] planned a lengthy investigation of the biological value of different proteins in the light of the new knowledge of their chemical structure. Their earlier work was carried out before the importance of accessory factors was recognised but they became aware from their experiments with individual proteins that some factor other than the supply of protein, salts and energy was complicating their observa-

tions. In the continuation of their researches, the results of which have appeared in some 30 papers in the *Journal of Biological Chemistry* from 1912 up to the present, the error due to absence of vitamins was obviated. Osborne and Mendel [1912-1920] confirmed and extended the observations of Willcock and Hopkins and showed that the addition of 3 % tryptophan to the zein given was sufficient to maintain rats over a period of 182 days, but that they failed to grow. When 2 % of lysine, in which zein is also deficient, was added, growth occurred. The problem of maintenance is therefore distinct from that of growth. The observations of Osborne and Mendel are some of the most important contributions to our knowledge of nutrition. The choice of rats enabled great numbers of experiments to be carried out and the extension of the periods of observation to cover a large fraction of the normal life of the animal. They prove that rats cannot supply some of the missing amino acids and that the minimum requirements and relative nutritive value of any particular protein depend upon the proportion of essential amino acids it contains. Their work also shows how a knowledge of the composition of particular proteins may be used for the economical adjustment of the nitrogenous portion of an animal's dietary by arranging that one protein shall compensate for the deficiencies of another.

The supplementary value of proteins from different sources has also been investigated by McCollum, Simmonds and Pitz, and McCollum, Simmonds and Parsons [1917 to 1921], whose observations, like those of Osborne and Mendel, were carried out upon rats. They found that cereal proteins could be satisfactorily supplemented by the proteins of milk, meat, kidney and casein and gelatin. Proteins of various leguminous seeds also usefully supplemented cereal proteins, *e.g.* wheat together with navy beans or peas.

The ultimate test of the nutritive adequacy of a protein is its capacity to nourish a young animal and provide for its complete growth and development and this, as far as the rat is concerned, is the criterion of the American investigators to which we have briefly referred. It may be surmised that, broadly speaking, conclusions arrived at from experiments on rats will be applicable in general to human nutrition. On the other hand the human mechanism may differ in detail. It will, for obvious reasons, be long before information as to the complete adequacy of individual proteins and quantitative data as to their biological values is forthcoming for human nutrition. In the meantime the findings of Osborne and Mendel, and McCollum and his co-workers have been applied with advantage to the feeding of stock.

From this more general survey of the subject we will now return to consider observations upon the minimum requirements for equilibrium when nitrogen is supplied in different forms. We have already referred to the observations of Rubner which led him to the conclusion that a different nitrogen minimum would be discovered for different proteins. This surmise was subsequently investigated in his laboratory by Karl Thomas [1909] who introduced the term "biological value." The expression "physiological value"

had been previously suggested by Voit and Korkunoff [1895] for a similar conception. Karl Thomas defined biological value as the number of parts of body nitrogen replaceable by 100 parts of the nitrogen of the foodstuff. Thomas's definition is not concerned with the relative digestibility of the protein. The replacement of the "Wear and Tear" quota was recognised as the only proper basis for comparison and in order to determine this value he fed himself on a carbohydrate diet (starch, sucrose, lactose) of high calorie value for periods of several days, during which the daily output of nitrogen in faeces and urine was determined. The figure to which this output fell was taken as his minimum requirements for the time being. During succeeding periods varying from one to four days a similar carbohydrate diet supplemented by a certain amount of the foodstuff under examination was taken and the N-intake and output determined as before. The N-intake was not as a rule kept constant and sometimes varied considerably on the different days. In most cases a negative N-balance was obtained. From the results Thomas calculated his biological value by three formulae based on the above definition, but differing from one another according to the way in which the nitrogen of the faeces is dealt with.

Some of Thomas's experiments lasted four to five weeks though no individual foodstuff was taken for longer than four days at a time. A period of one or two days on nitrogen-free diet was usually interposed between the experiments. Sixteen foodstuffs were investigated and their biological values recorded. These varied from 100 % in the case of milk to 30 % in the case of maize. We shall have occasion to discuss some of his results after dealing with our own experiments.

Shortly before this work of Thomas appeared the results of experiments upon dogs with a similar object were published by Michaud [1909]. The output of nitrogen on diets of dog-flesh, sugar and fat was compared with that on diets of horse-flesh, caseinogen, gliadin, and edestin and on the carbohydrate and fat alone. Nitrogenous equilibrium was attained with an amount of nitrogen in the form of dog-flesh equivalent to the nitrogen output on the nitrogen-free diet. Negative balances were obtained with the other proteins, the greatest being in the case of gliadin and edestin.

Zisterer [1910] found differences between caseinogen, flesh and gluten. These were however, in his opinion, too small to have practical significance.

Observations upon pigs were made by McCollum [1911]. These animals lend themselves to metabolism experiments of this kind as they will consume sufficient of a diet free from nitrogen to obtain the necessary calories over a considerable period. Their minimum nitrogen expenditure can therefore be determined with reasonable accuracy.

After a period of a week upon a diet of starch alone, the animals were fed for several days with the same ration to which was added a small amount of gelatin, zein, caseinogen or other protein. This was followed by the starch ration for a further period of some days. An amount of nitrogen in the

form of gelatin equal to that of the urine upon the starch diet was found to cover 39 % and in the form of zein 73 % of the animal's expenditure. The same amount of nitrogen in the form of cereal protein did not cause any rise in the nitrogen of the urine and with caseinogen the rise was small. McCollum's experiments seem to avoid all the obvious pitfalls and his results indicate a much higher biological value for cereal proteins when fed to the pig than those arrived at by Thomas's experiments upon himself.

Hindhede [1913, 2] concludes that nitrogen equilibrium may be attained on a diet of potatoes and margarine containing only 20 g. of digestible protein. The figure is, however, arrived at by deducting the nitrogen of the faeces from the intake. This method of calculation is not in accordance with our knowledge of the origin of a considerable portion of the faecal nitrogen and will furnish a too favourable balance sheet.

Hindhede [1914] vigorously contests the findings of Rubner and Thomas and claims to have attained nitrogenous equilibrium on as small an amount of protein in the form of bread as of potatoes. He declares as a result of his lengthy experiments that the proteins of potatoes, bread and meat can replace those of the body gram for gram. With Hindhede's criticisms of some of Thomas' experiments and treatment of his data, we are, for the most part, in agreement but must at the same time admit the justice of a great part of Rubner's equally severe criticisms of Hindhede's evidence, in particular, as regards the justification for assuming that all the nitrogen of the faeces represents undigested food proteins.

Abderhalden, Fodor and Röse [1915] carried out some experiments to determine the minimum requirement of nitrogen in the form of different kinds of bread and potatoes. The subject of the experiments was Hofrat Röse who possessed some peculiarly advantageous characteristics. Röse was accustomed to a monotonous diet, neither smoked nor drank alcohol and was in the habit of chewing his bolus 120 times before swallowing it. Experiments of three to eight days' duration were made on diets of potatoes, white wheaten bread, Swedish bread and kommiss brot, the last two being made from rye and containing bran. The experimental facts seem to us to warrant the conclusion that a gross intake of 4.5 g. of potato nitrogen, equal to 0.074 g. N per kilo, were adequate in the case of this Hofrat who chewed so long and so well. 9 g. N in the form of white wheaten bread was not quite sufficient and 10.8 g. N as supplied in the rye bread was only just enough to reach equilibrium. This is not, however, the interpretation placed upon the results by Abderhalden and his co-workers, who conclude that bread nitrogen is as good as potato nitrogen and that for both of them the minimum nitrogen requirement is round about 4 g. for a man of 60 kilos.

Rubner [1919] in the course of some studies of the capacity of certain vegetable nutriments to satisfy nitrogen needs, undertaken during the war, investigated different sorts of bread and the effect of milling to varying extent on the value of the product as a source of nitrogen. The paper covers a good

deal of ground and contains some particularly useful experiments with white wheat bread which can be compared with our own upon whole wheat. The bread was made, in one series, from white flour, 30 % milled, in the other of the same flour mixed with rye-bran to the extent of 30 %, so-called "Finkler brot." 10 g. of the N as contained in the fine flour and between 10 and 11 g. of that in the Finkler bread were adequate to maintain equilibrium.

Recently Sherman and his co-workers [1918, 1 and 2, 1919, 1920] have obtained results which are difficult to harmonise with those of Thomas. In experiments upon men and women, nitrogenous equilibrium was attained with an intake of 0.08 g. N per kilo, nine-tenths of this being supplied by cereal proteins and one-tenth by those of milk or apple. Wheat, maize and oats were found of equal value as a source of nitrogen and the view is taken that these cereal proteins possess a higher biological value than Thomas found. The effect of the supplementary action of the small quantity of milk may, in the light of the observations of Osborne and Mendel [1917] and of McCollum, Simmonds and Parsons [1921] be considerable.

Boruttau [1915] believes that the low value of cereals as a source of nitrogen is greatly improved when these are consumed without the removal of the bran, etc. The biological value of 145 % he obtained for the nitrogen of bran, is, in our opinion, an instance of the misuse of arithmetical formulae.

R. O. Neumann [1919] made an excellent experiment upon himself in 1917 in which he lived *exclusively* on rye bread, cane sugar and water for 40 days. Nitrogenous equilibrium was attained with 1000 g. bread and 300 g. cane sugar (= 9.9 g. N). The total calories of this diet amounted to 3630 or 63.8 per kilo. On raising the calorie value of the intake to 4434 (or 73 per kilo) the nitrogen excreted steadily fell to 7.3. This indicates that Neumann in a long continued experiment could maintain nitrogenous equilibrium on less than 7 g. of nitrogen in the form of rye-proteins if excess of calories were furnished by sugar. The experiment is also interesting as indicating the sensitiveness of the nitrogen balance to the addition of carbohydrate. This aspect of the experiment will be discussed later.

From a survey of the literature it is clear that certain of the proteins possess very different biological values both for growth and maintainance. There is, however, much uncertainty as to the degree to which the admixed proteins occurring in individual foodstuffs, where one protein to some extent complements the deficiencies of another, vary in value as a source of nitrogen.

The divergence of opinion is most marked when it is based upon metabolism experiments on man over limited periods.

OUR OWN OBSERVATIONS.

INTRODUCTORY.

We commenced our investigation lightheartedly with the comparatively modest object of re-determining the relative values of certain cereal proteins in human nutrition, in particular that of maize, in view of the significance given by Goldberger and others [1915, 1920] and Wilson [1921] to the low biological value of maize in the causation of pellagra. The difficulties in arriving at values which could justifiably be compared were soon, however, apparent and it became essential to investigate thoroughly the conditions under which valid results might be obtained. In so far as the problem can be solved by metabolism experiments on adult animals the one unexceptional way to determine the relative biological values of proteins would be to ascertain the minimum intake on which nitrogen equilibrium can be maintained in each case. This sounds simple but unfortunately a positive balance only tells one that the intake is sufficient but not how much it is in excess and a number of experiments have to be performed to ascertain the minimum quantity.

We were ourselves the subjects of the experiments. This is inconvenient but advantageous, for the experiments are exacting and necessitate constant supervision of one's actions if sources of error are to be avoided. The partial abandonment of the joys of life is to some extent compensated by interest in the results.

Nevertheless, the unnecessary multiplication of irksome experiments on one's self, each extending over many days, is a thing to be avoided and it would be very desirable if a couple of observations could be made and the minimum requirements calculated from these with sufficient accuracy. This is what Thomas attempted to do. But in adopting such a method an assumption is made, the truth of which is by no means self-evident, namely, that the value of any protein for biological purposes remains uniform whatever the amount taken. The assumption would be justified if the nitrogen were utilised in the first instance to form some complex, such as "Vorratseiwiss."

In this case the biological value, as pointed out by Abderhalden, would be determined by the ratio of the percentages, in food protein and body complex respectively, of that amino acid for which this ratio has the lowest value, unless the body has the capacity to synthesise that particular amino acid from others.

It might also be true if the nitrogen requirements are of varying nature so long as they are also indivisible, that is that no single requirement can be satisfied unless at the same time all the others are satisfied.

The former of these two conceptions would appear to have been accepted without question by Rubner and Thomas though the case of gelatin obviously could not be treated in this way. Gelatin was considered to be capable of sparing body protein to the extent of 30-40 % when fed in relatively small

amounts but unable to do more than this however much was taken. Its biological value, if calculated by any of Thomas's formulae would therefore appear quite appreciable when the intake was small but almost zero if the intake was very large. Yet, Boruttau [1919] has actually made use of these formulae to calculate the biological value of gelatin and has obtained a result of 58.2 %.

Another possible disturbing factor (which we have reason to suppose occurs) is the varying economy with which the body deals with the amino acids supplied to it, according to their abundance.

The various possibilities stated above may be made clearer by a diagram in which abscissae represent real nitrogen intake and ordinates the real nitrogen output.

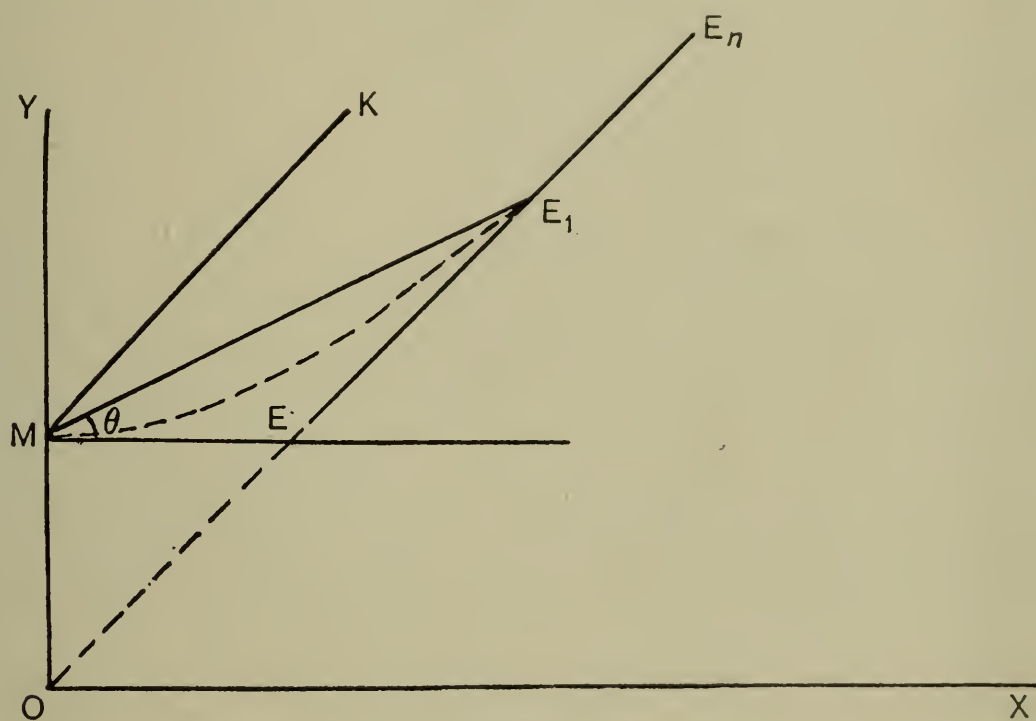


Fig. 1.

Let $OM (= m)$ be the output on a N-free diet of adequate fuel value. Then m is equal to the nitrogen minimum.

Suppose that an ideal protein (B.V. = 100) is fed in gradually increasing amounts and is utilised without waste. So long as the intake remains lower than m the output will remain constant and equal to m since the food protein saves an equal amount of body protein. The graph of intake and output will therefore be a line parallel to the x -axis and at E where $ME = MO$ the body will be in nitrogen equilibrium.

If now the intake be further increased, equilibrium will again result (unless the body is in a growing condition or has been previously starved of N) and the graph will now follow the line EE_n at an angle of 45° to the axis.

In the case of a protein of value less than that of the ideal protein just considered, equilibrium will not be attained on an intake equal to m but on

some greater amount e_1 (at the point E_1). On all amounts less than this, the output will exceed the intake and the graph will follow some line joining ME_1 . Whether this line is straight or curved will depend on the conditions set out above, viz.:

- (1) indivisibility of the nitrogen requirements of the body;
- (2) uniform economy with varying nitrogen intake.

If these conditions are obtained the line ME_1 will be straight and its equation will be $y = m + x \tan \theta$ where y is the real output corresponding with any real intake x less than e_1 .

For higher values of x the graph will follow the line E_1E_n .

Thomas's formulae can be very simply expressed in terms of θ ; thus formula B

$$\begin{aligned} \text{B.V.} &= 100 \frac{\text{Urine N in N-free diet} + \text{faeces N} + \text{balance}}{\text{N-intake}} \\ \text{becomes} \quad \text{B.V.} &= 100 \frac{m + (x - y)}{x} \\ &= 100 \frac{m + x - (m + x \tan \theta)}{x} \\ &= 100 (1 - \tan \theta). \end{aligned}$$

If the above conditions do not obtain, *e.g.* if a number of different amino acids are required for specific purposes, which are distinct and can be separately satisfied, the graph of a protein rich in certain of these acids but poor in others would be a curved line such as the dotted line joining M and E_1 in the diagram. This curvature would express the fact that a certain fraction of the body's needs could be satisfied by a smaller amount of this protein than would correspond with the amount required to obtain equilibrium. The angle θ and the biological value would then vary for different values of x .

The graph of a protein, unable by itself to satisfy any portion of the body's nitrogen requirements, would be a straight line MK parallel to OE_n , since the nitrogen output would always be equal to the intake + m . For this line $\theta = 45^\circ$ and the equation $y = m + x \tan \theta$ becomes $y = m + x$ while the biological value = $100 (1 - \tan 45^\circ) = 0$.

In our opinion there was very little reason for assuming that these graphs would necessarily prove to be straight lines. It is true that Thomas calculates his values from individual daily balances and takes the average of the results, but such daily balances are too variable and are subject to too great experimental errors to offer any satisfactory proof of the uniformity of the value. We therefore set out to obtain evidence on this question by determining as accurately as possible a number of points for the same protein but for different values of x . Our results will be considered later but we may here state that in the case of bread proteins the points do lie on, or close to, a straight line. In the experiments with nitrogen in the form of milk results were at first obtained indicating pronounced curvature of the line, and nitrogen equilibrium was not obtained until more than 11 g. of milk nitrogen was taken per day. By increasing the amount of carbohydrate however, so that the

fuel value of the diet was greatly in excess of requirements and the respiratory quotient greater than 1, equilibrium was finally reached with half this amount, and bearing in mind that when x , the intake, is very small, the physiological errors of experiment become relatively great, the observations could now perhaps be expressed by a straight line. As long as any doubt exists of the rectilinear character of the line ME it will obviously be prudent to place reliance only upon observations in which x and y are as large as possible short of equality.

FACTORS WHICH MUST BE CONSIDERED IF VALID RESULTS
ARE TO BE OBTAINED.

1. *The time required to reach a uniform N-output on a constant intake.*

The effect of the previous diet upon the N-output and the length of time required to reach a constant output on a constant intake which is either greater or less than that of the preceding period, was clearly demonstrated by the old experiments of C. Voit [1866, 1867]. The N-output of a dog during the first days of starvation varied with the amount of protein in the previous diet but fell gradually until a relatively constant figure was reached on the fifth or sixth day. When the dog was given a constant meat diet for some days and then a considerably greater (or less) amount daily during a further period a similar gradual increase (or decrease) in the N-output was observed during five or six days before equilibrium again set in at the new level. These observations have been repeatedly confirmed by Grubner [1901], Landergren [1903], Kinberg [1911] and many others.

The rapidity with which the nitrogen excretion diminishes obviously depends on the difference between the N-intake during the experimental and the preceding periods and will be greatest when a period of nitrogen starvation follows one of high protein intake or *vice versa*. There is no reason to suppose, however, that this gradual change is ever replaced by an immediate jump to the new level even when the difference between the two planes of N-intake is but small, though naturally the absolute amounts of the variations will be correspondingly less.

Whether the N-output is also influenced by the nature as well as the amount of the protein taken in the foregoing period is more difficult to decide. If part of the nitrogen of the previous diet is stored up in any form that can be utilised by the body (*e.g.* amino acids) and not merely in the form of unexcreted end products, we should expect the N-output during the first few days of the succeeding period to be influenced thereby—unless during both periods the body is in N-equilibrium. For example if the diet during the first period contains 10 g. of N from caseinogen, and during the second period a negative balance occurs on 10 g. of N from zein, the amount of this negative balance might very well be less during the first few days of the zein diet than on the latter days of the same period owing to the supplementary action of amino acids stored up during the caseinogen diet. The results of experiments [Robison, 1922] in which a diet containing gelatin as the sole protein followed

a diet of mixed proteins suggest that this does occur, and that therefore when the diet is changed in any way—either in amount or nature of the protein—the N-output cannot be considered to represent that of the second diet until some days have elapsed.

It follows that metabolism experiments are subject to error from these causes and that the error diminishes as the duration of the experiment increases. As a compromise, we have excluded from calculation the figures for the first three or four days in arriving at the N-output. It is also advisable that the N-intake should not vary during the experiment. Most of Thomas's experiments are subject to both these sources of error.

2. *The time required for the elimination of errors due to the fluctuation in the N-output.*

The very considerable fluctuations that may occur in the amount of nitrogen excreted in the urine by men receiving an absolutely constant diet have been noted by Bornstein [1898], Atwater and Benedict [1902] and by Falta [1906], by all of whom the cause was considered to be psychical. Atwater and Benedict also noted the increased diuresis which often accompanied a high N-output and thought it probable that the diuresis was the direct result of the psychical stimulus and the cause of the increased N-elimination. Falta observed variations of 4–5 g. in the N-output on individual days although in equilibrium over the period as a whole.

Neumann [1899] studied the influence of variations of the urinary flow upon the daily output of nitrogen, the intake remaining constant. When diuresis was produced by increasing the water drunk from one to three litres, the N-output increased from 10.5 to 14.3 g. and did not reach the original level until the third day.

In almost all our experiments such fluctuations, amounting frequently to 25 % of the mean output, have occurred and not least in those experiments in which the most rigorous attention has been paid to constancy of diet and fluid intake, and to regularity in the mode of life.

At first we also were disposed to attribute these fluctuations to increased diuresis but the frequent lack of any correlation between the two compelled us to modify our opinion. Some factors (*e.g.* mental strain or excitement) may possibly affect both diuresis and N-output, but the latter does not always coincide with the increased volume of urine and may even vary in the opposite sense. In one experiment, for example, the minimum N-output corresponded with the maximum volume of urine.

Judging from the experiments of Voit and other workers on dogs and of McCollum on pigs, it would appear that in the case of these animals the fluctuations in N-output on a constant diet are usually less considerable than with man. It is clear that calculations based upon the nitrogen balance sheet for single days, a procedure frequently resorted to, are subject to very large errors and that these can only be eliminated by taking the average results over a number of days.

3. *The necessity for abundant energy supply.*

It has been universally recognised that proteins will be used as fuel unless an adequate supply of fat and carbohydrate is provided, but there have been different opinions as to what constitutes adequacy in this respect. In their search for the protein minimum some of the earlier workers considered it necessary to supply a diet of fuel value very greatly in excess of the energy requirements of the organism. Thus in Klemperer's [1889] experiments on two young men, a diet of 5020 calories, equal to about 75 calories per kilo was given. Sivén, however, was of the opinion that such excess was unnecessary and that the minimum could be reached without increasing the calorie value of the diet above the normal. The fuel value of Sivén's diets was equal to about 40 calories per kilo body weight.

Hindhede's [1913, 2] attitude is somewhat difficult to understand. He considers that an abundant calorie intake is necessary if the protein minimum is to be attained, but that this minimum will vary with the calorie value of the diet. He does not believe that a quiet old man, for whom a diet of 1500–2000 calories is sufficient, can have the same minimum as an active young man who requires a diet of 3000–5000 calories. From the results of his experiments, he calculates by simple proportion, the minimum for a standard diet of 3000 calories. As on a diet of 3900 calories F. Madsen's minimum was equal to 25 g. of digestible protein so, according to Hindhede, for 3000 calories the minimum would be 19 g. of digestible protein.

Rubner [1919] has criticised this procedure, and in our opinion justly, on the ground that it is unwarranted by the facts, and considers that the values so calculated to 3000 calories possess no scientific basis.

Rubner's own conclusions are that the N-minimum may sometimes be reached when no more than a third of the total energy requirements are satisfied, in other cases only when they are fully met, while in others a diet considerably in excess of these requirements will be necessary. These differences he considers are due to the varying nutritional condition of the body cells. The minimum is, however, most easily reached on an abundant carbohydrate diet.

In our own experiments on a diet nearly N-free we appeared to reach our N-minimum with an intake of 45–50 calories per kilo body weight of which about one-third was taken in the form of fat, whereas on a diet of milk (with additional carbohydrate), equilibrium was not readily obtained until the fuel value was increased to about 55 calories per kilo of which only 10 % was in the form of fat.

The effect of diets containing varying proportions of fat and carbohydrate on the protein minimum has been studied by Zeller [1914], who found that the mixed diet was just as efficacious in reducing the consumption of body protein as one of carbohydrate alone, so long as the proportion of carbohydrate to fat did not fall below 1 : 4.

Neumann's [1919] experiment upon himself, with a diet composed of bread and sugar, affords a striking demonstration of the effect of excess of carbohydrate in lowering the protein minimum. It is obvious that Prof. Neumann readily stores fat. Otherwise, he could not consume 73 calories per kilo over a period of three weeks, unless doing hard work. In his case, presumably, a greater excess of carbohydrate would be required to maintain the blood sugar at a high level than in our own, owing to the greater greed of his connective tissue cells.

It would seem, therefore, that the most certain way of determining the protein minimum would be to take a diet consisting mainly of carbohydrate and so much in excess of the energy requirements that the blood sugar is maintained high, the liver and muscles are kept well stocked with glycogen and the surplus is being stored as fat, as indicated by a respiratory quotient above unity. This was accomplished in the latter part of our experiment on milk. It is by no means easy for one of the meagre habit of the subject of the experiment (C.J.M.) as it increases the distaste for the sufficiently unappetising ration of starch and lactose and if persisted in too enthusiastically it produces unpleasant symptoms.

4. *Reduction of the nitrogen in the basal diet to a minimum.*

The carbohydrate (starch, lactose, etc.) and fat, etc. which form the basal diet for these experiments are nominally but not absolutely nitrogen-free. The amount of nitrogen taken in this form can of course be estimated but its biological value is unknown and this complicates the results. It is therefore important to reduce the nitrogen in the basal diet to a minimum by careful selection of the most suitable forms of such foods.

Most observers have neglected to take account of the nitrogen in the starch, etc. fed. As large quantities of such basal ration are consumed it may not be negligible in the case of experiments in which small quantities of some protein are being given.

5. *Accessory food factors and inorganic salts.*

The duration of metabolism experiments is limited by the difficulty of providing an adequate supply of the accessory food factors. The ill effects of long continued low-protein diets observed in some of the older animal experiments was no doubt sometimes due to the deficiency of one or other of these factors.

Fat soluble *A* can be introduced in the form of rendered butter fat or cod liver oil, and water soluble *C* in the form of lemon juice, the nitrogen content of which is very low, but we have found no method of introducing the water soluble *B* without an undue amount of possibly very valuable nitrogen.

When the diet is deficient in inorganic salts or is such as to afford an acid ash, adjustment by suitable amounts of a salt mixture is essential. McCollum and Hoagland [1913] found that the endogenous metabolism of the pig reached its lowest level when the animal was given an abundant carbohydrate diet together with a salt mixture of an alkaline character. When an acid salt mixture was given the urinary output rose, the increase occurring in the amount of ammonia. They concluded that this animal is not able to use the nitrogen of the urea fraction for the neutralisation of acid.

6. *The apportioning of the nitrogen in the faeces.*

The difficulty of correctly apportioning the nitrogen in the faeces to unabsorbed food nitrogen and excretion from the alimentary tract respectively, is the limiting factor in most experiments in which the total intake of nitrogen is small, and the way in which different observers treat the faecal nitrogen has given rise to much controversy and recrimination.

The daily nitrogen output in the faeces on a protein-free diet usually amounts to about 1 g. On other diets the amount may be considerably greater than this and the question arises—what is the significance of this excess? Does it represent unabsorbed food residues or increased loss of body nitrogen? This difficulty was recognised and discussed by Karl Thomas, whose three formulae for the calculation of the biological value of protein differ only in the assumptions that are made with regard to this point. In formula A the whole of the nitrogen of the faeces is assumed to represent unabsorbed food; in formula B it is assumed to arise entirely from the body, while in formula C 1 g. N (which is taken as the average output on protein-free diet) is assumed to be body nitrogen, any excess over this amount being ascribed to unabsorbed food.

Rubner [1915] has investigated this problem in connection with his researches on the digestibility of various foodstuffs. He has devised a method for estimating the amount of nitrogen in the faeces present in the form of undigested food residues (vegetable cell membranes) by making use of the insolubility of the latter in acid alcohol and in a concentrated solution of chloral hydrate in which he states bacteria, epithelial cells, etc. are dissolved. He considers that the rest of the nitrogen comes from the body and represents metabolic products, and he concludes that such body nitrogen forms a very considerable proportion of the increase in the total nitrogen of the faeces that commonly occurs when the diet consists largely of whole cereals, vegetables or fruit.

Rubner found that on a vegetable diet nearly half the total nitrogen of the faeces was soluble in acid alcohol whilst on a diet consisting chiefly of animal foodstuffs the proportion of soluble nitrogen was still greater. We have obtained similar results with faeces resulting from a mixed diet, but do not

know in what form the whole of this soluble nitrogen is present, and are therefore not able to draw definite conclusions as to its origin.

So long as this uncertainty remains, the nitrogen of the faeces will be the limiting factor for the accuracy of such experiments as those here described. We have therefore followed Thomas's plan and have calculated our results in two ways; the first, assuming that the whole of the nitrogen of the faeces comes from the body and that this amount plus the urine N on N-free diet represents the body's minimum requirements under the conditions of the experiment; the second, assuming that the amount by which the nitrogen of the faeces exceeds the average amount excreted on a N-free diet represents unabsorbed food and is therefore to be subtracted from the total intake in order to arrive at the true nett intake, *i.e.* the absorbed nitrogen. The truth will probably lie somewhere between these two extremes.

EXPERIMENTAL.

Firstly as to general procedure. The N of all foodstuffs and beverages used was determined. The food was weighed in the same condition as that in which the N was determined and the total intake of N recorded in the tables can be relied upon to plus or minus 0.01 g. The urinary excretion of each 24 hours was collected in the presence of toluene, weighed, and duplicate determinations of the N content made. Care was exercised to see that invalid sampling from the deposition of either urates or ammonium magnesium phosphate did not occur. The faeces, after collection each day, were mixed with some H_2SO_4 to prevent decomposition and possible loss of NH_3 and great care was taken at the end of each period to ensure the validity of the samples taken for analysis. N determinations were made upon about 10 g. in duplicate, only closely concordant results being accepted.

The evacuation of the intestines is not usually so regular and complete that any great value can be placed on the figures for the nitrogen excretion on individual days. The best that can be done is to determine the nitrogen in the faeces for the whole experimental period and from this to calculate the average daily output. The latter may vary within rather wide limits even when the diet contains little or no nitrogen, and appears to depend to some extent on the bulk and character of the faeces. In order that these might be kept as uniform as possible agar-agar was taken when the diet consisted wholly or largely of completely digestible foodstuffs.

Food was taken in approximately equal amounts three times a day at the customary hours, and we led our usual life. In the experiments upon milk we abandoned all attempts to make our basal ration of fat and carbohydrate resemble a repast and drank a suspension of uncooked corn-starch in a saturated solution of lactose. This was followed by an alkaline salt mixture and 2 g. of agar-agar. In this way 600 calories were contained in about a tumbler full. Microscopical examination of the faeces showed that the

starch was completely digested. When the starch-lactose mixture exceeded 250 g. at one meal some glycosuria occurred temporarily. On the N-minimum experiment the faeces were olive green as the bile pigment was unreduced. Little gas was formed.

The following are the essential data regarding the subjects of the experiments, ourselves:

C.J.M. Age 56. Weight 61 kilo. Height 183 c. Very thin, stores fat with difficulty. Mode of life: laboratory work. Exercise: lawn tennis before breakfast for three-quarters of an hour and about three miles walk during the day.

R.R. Age 37. Weight 59 kilo. Height 173·5 c. Spare, does not store fat readily. Usual mode of life consists chiefly in laboratory work. Very little regular exercise beyond daily walk of two to four miles.

Minimum nitrogen expenditure on carbohydrate-fat diet.

Our minimum nitrogen expenditure was determined in two experiments, during which our diet was as nearly as possible nitrogen-free. In the vain attempt to make this diet appetising much labour was expended in endeavouring to prepare the food in a varied and attractive manner. Biscuits made of corn starch with 20 % of fat proved quite palatable when taken in small quantities but nauseating in bulk. A biscuit, whose chief defect was its hardness, made from starch, dextrin and a little fat was finally adopted and formed, with butter and honey, the chief article of diet. A starch mould flavoured with lemon juice was also taken. Weak tea with lemon and sometimes black coffee and a little vermouth was drunk. After the first few days of this diet no desire was felt either for this or for any other food, nor did the sight of our first normal meal at the close of the experiment arouse any appetite. The drinking of a glass of hot milk, however, excited in a few minutes a very keen appetite and desire for food. The quantities of the individual constituents of the diet varied somewhat from day to day but were always accurately measured and noted. Those for a single typical day are set out in Table I while the total nitrogen intake and fuel value of the diet for each day are shown in Table II in which are also set out the daily output of nitrogen in the urine and faeces and the nitrogen balance. In this and in all other experiments the nitrogen in the tea and coffee has been assumed to consist chiefly of caffeine and to be excreted unchanged in the urine. It has therefore been subtracted in all cases from the total intake and from the urinary nitrogen output. Any error involved in this method of treatment must be of negligible dimensions.

The nitrogen in the urine fell steadily until the last day of the experiment when a rather considerable rise occurred in the case of both C.J.M. and R.R. This is probably to be explained by the fact that, owing to the difficulty of consuming the food when all appetite was in abeyance, the fuel value of the diet was reduced during the last day or two.

Table I.

Foodstuff	N-minimum 2.		Diet R.R. 4. xii. 20		
	N per 100 g.	Calories per 100 g.	Weight g.	N g.	Calories
Corn starch	0.042	360	280	0.118	1008
Dextrin	0.065	360	50	0.033	180
Butter	0.080	775	90	0.072	698
Margarine (rendered)	0.010	900	15	0.002	135
Honey	0.023	327	55	0.013	180
Sucrose	—	395	25	—	99
Lactose	0.013	370	65	0.008	241
Lemon juice	0.067	40	30 cc.	0.020	12
Vermouth	0.005	140	25 "	0.001	35
Tea infusion	0.006	—	1200 "	0.072	—
Agar-agar	0.242	—	15 g.	0.036	—
Salt mixture	—	—	5 "	—	—
Total				0.375	2588
Excluding tea N				0.303	45 per kilo

Table II.

N-minimum 1. Subject: R.R. Fluid Intake: 2000-2500 cc.

Date 1920	Body weight <i>k</i>	Daily Intake			Output			Balance N g.
		N (excluding tea and coffee) g.	N tea and coffee g.	Calories per kilo	N urine g.	N faeces g.	N total g.	
Nov. 15	58.5	0.30	0.16	56	8.64	—	—	—
" 16		0.28	0.06	54	5.31	1.05	6.36	-6.08
" 17		0.30	0.15	55	3.63	"	4.68	-4.38
" 18		0.25	0.10	51	2.66	"	3.71	-3.46
" 19		0.22	0.06	43	2.25	"	3.30	-3.08
" 20	57.7	0.23	0.15	49	2.82	"	3.87	-3.64
	(21. xi)							

N-minimum 2. Subject: R.R. Fluid Intake: 4000 cc.

" 29	58.6	0.27	0.07	49	8.79	1.13	9.92	-9.65
" 30	(23. xi)	0.30	0.07	50	4.71	"	5.84	-5.54
Dec. 1		0.30	0.08	51	3.46	"	4.59	-4.29
" 2		0.35	0.08	51	2.71	"	3.84	-3.49
" 3		0.32	0.09	52	1.99	"	3.12	-2.80
" 4		0.30	0.07	45	2.17	"	3.30	-3.00
" 5	57.7	0.27	0.06	44	2.01	"	3.14	-2.87
Average of last 3 days		0.30	0.07	—	2.06	1.13	3.19	-2.89

N-minimum 1. Subject: C.J.M. Fluid Intake: 2000 cc.

Nov. 14	61.7	0.33	0.20	56	8.61	—	—	—
" 15		0.30	0.20	47	4.89	1.24	6.13	-5.83
" 16		0.30	0.20	47	3.28	"	4.52	-4.22
" 17		0.38	0.21	52	3.28	"	4.52	-4.14
" 18		0.32	0.21	46	2.48	"	3.72	-3.40
" 19		0.30	0.21	45	2.25	"	3.49	-3.19
" 20	60.9	0.21	0.18	35	2.49	"	3.73	-3.52
	(21. xi)							

N-minimum 2. Subject: C.J.M. Fluid Intake: 4000 cc.

" 29	61.9	0.34	0.31	59	8.89	—	—	—
" 30		0.34	0.27	55	4.78	—	—	—
Dec. 1		0.34	0.33	55	3.40	1.17	4.57	-4.23
" 2		0.33	0.32	53	2.41	"	3.58	-3.25
" 3		0.35	0.32	52	2.51	"	3.68	-3.33
" 4		0.35	0.29	52	2.40	"	3.57	-3.22
" 5	60.5	0.33	0.26	48	2.13	"	3.30	-2.97
Average of last 3 days		0.34	0.29	—	2.34	1.17	3.51	-3.17

On plotting the amounts of urine nitrogen as ordinates against the time in days as abscissae it became apparent that all the points except the last would lie on, or close to a curve for which a simple logarithmic expression was found (Figs. 2 and 3). Among several possible interpretations of this curve is the simple one that the falling nitrogen output represents washing out of some metabolic products from the tissues, the amount washed out each day being proportional to that still present.

A second similar experiment was therefore carried out in order to confirm this result and also to discover whether the steepness of the curve could be altered by drinking large quantities of water and thus greatly increasing the volume of the urine. Apart from the volume of total fluid, which was doubled, the diet did not differ from that taken in the first experiment. The results are set out in Table II.

The regularity in the fall of the nitrogen output was again observed but the change in the rate of this fall was very small.

The average outputs in urine and faeces on the last three days of this experiment have been taken as representing the minimum nitrogen expenditure on such a diet. Whether this amount also represents the minimum expenditure on a diet that is absolutely nitrogen-free will depend on the biological value of the small amount of nitrogen in the food consumed during the above experiment. If this has a value of 100 %, *i.e.* if it can replace and therefore spare an equal amount of body nitrogen, the output thus determined will be equal to the real minimum expenditure. If the value of the food nitrogen is zero, *i.e.* if it is unable to satisfy any fraction of the body's requirements, it must be excreted in addition to the amount representing the latter and the minimum expenditure will therefore be equal to the observed output less the full amount of the nitrogen intake. Probably this nitrogen, which was present chiefly in the corn starch and butter, has a value intermediate between 100 and zero. The minimum nitrogen expenditure will therefore be some amount between those shown in the last two columns below.

<i>Minimum nitrogen expenditure.</i>				
Subject	Urine g.	Faeces g.	Total output g.	Total output - intake g.
C.J.M.	2.34	1.17	3.51	3.17
R.R.	2.06	1.13	3.19	2.89

THE BIOLOGICAL VALUE OF THE PROTEINS OF WHOLE WHEAT.

A series of experiments was carried out with whole wheat flour with two objects in view:

- (1) to determine the Biological Value of the wheat proteins from the minimum amount with which nitrogen equilibrium can be attained;
- (2) to discover whether the Biological Value is uniform for varying amounts of wheat nitrogen.

The first of these has been investigated upon man by other workers, whose

results will be considered with our own. The great practical importance of this question and the astonishing divergence between the results of previous investigations were sufficient reasons for further study.

Method of experiment.

The large variations in the percentage of water, and consequently of nitrogen, in different parts of a loaf of bread and the difficulty of obtaining a satisfactory sample, render it impossible to estimate the total nitrogen content of the loaf with sufficient accuracy for these experiments. We decided therefore, to base our calculations on the flour and to bake the bread ourselves. By suitable manipulation it was found possible to prepare loaves from 500 g. of flour with a maximum loss of less than 0.1 %. The other materials used were butter (5 %), salt, baking powder (prepared from tartaric acid, sodium bicarbonate and corn starch) and water. The loaves were baked in the laboratory for about one hour at a temperature of 240°–250°, and were only very slightly browned so that no appreciable loss of nitrogen can have occurred during the baking. For the first period, a somewhat coarsely ground whole wheat flour containing 1.85 % N was used but on increasing the daily ration from 300 g. to 450 g., considerable discomfort was experienced from the large particles of bran, and the bread was poorly absorbed. For the second and remaining periods a very finely ground flour prepared from a mixture of English and foreign whole wheat was employed. We found it very palatable and well absorbed, as the figures for the nitrogen in the faeces indicate. Only when the daily consumption had been raised to 550 g. and the total calories to 63 per kilo did we experience any discomfort.

The experiment was carried out in duplicate on ourselves and commenced with a total nitrogen intake of nearly twice the amount of our minimum requirements. On this diet a considerable negative balance occurred and the amount of wheat nitrogen was therefore increased during successive periods until equilibrium was finally attained.

The daily ration of flour was kept constant during each separate period of the experiment, but some latitude was permitted in the amounts of the remaining constituents of the diet. The actual quantities taken were, however, measured and the variations in any one period were not such as to affect appreciably the total nitrogen intake or greatly alter the fuel value of the diet. The diet set out in Table III shows the average amounts consumed by R.R. from the 24th to the 31st of October but except for the quantity of flour it would with slight variations serve for the whole experiment.

The experimental results are set out in Tables IV and V.

As in the previous experiments it has been assumed that the nitrogen consumed in tea and coffee would be excreted unchanged and the amount has therefore been subtracted from both intake and output (urine N).

During certain periods indicated by the letter (A) in the first column of

Table III.

Foodstuff	Whole wheat.		R.R. Diet during period 5, 24-31 Oct. 1920		
	N per 100 g.	Calories per 100 g.	Weight g.	N g.	Calories
Flour, whole wheat	2.162	360	450	9.730	1620
Corn starch	0.042	360	36	0.015	130
Butter	0.080	775	56	0.045	434
Dripping	0.016	885	80	0.013	708
Honey	0.023	327	40	0.009	131
Marmalade	0.052	341	20	0.010	68
Cane sugar	—	395	56	—	221
Lemon juice	0.067	40	20 cc.	0.013	8
Tea	0.006	—	960 "	0.058	—
Coffee	0.041	—	100 "	0.041	—
Vermouth	0.005	140	25 "	0.001	35
Total					9.935
Excluding tea and coffee					9.836
					3355
					58 per kilo

Table IV.

Diet: whole wheat. Subject: C.J.M.									
Period	Date 1920	Body weight <i>k</i>	Daily Intake			Daily Output			Balance N g.
			N bread g.	N total g.	Calories per kilo	N urine g.	N faeces g.	N total g.	
1. (A)	Sept. 30-Oct. 2	61.80 (26. ix)	5.55	5.81	46	—	—	—	—
(O)	Oct. 3-5	61.50 (3. x)	"	5.70	44	5.62	2.14	7.76	- 2.06
2. (A)	" 10-14	60.45 (10. x)	8.32	8.63	56	—	—	—	—
	" 15	"	"	"	"	8.26	2.17	10.43	- 1.80
	" 16	"	"	"	"	7.62	"	9.79	- 1.16
	" 17	61.44	"	"	"	6.98	"	9.15	- 0.52
	Average		8.32	8.63	56	7.62	2.17	9.79	- 1.16
3. (A)	" 18		8.65	8.97	57	7.86	2.35	10.21	- 1.24
	" 19		"	"	"	7.25	"	9.60	- 0.63
	" 20		"	"	"	7.15	"	9.50	- 0.53
	Average		8.65	8.97	57	7.42	2.35	9.77	- 0.80
4. (A)	" 21	60.65	9.73	9.98	53	7.25	1.74	8.99	+ 0.99
	" 22	"	"	"	"	7.92	"	9.66	+ 0.32
	" 23	61.03	"	"	"	7.30	"	9.04	+ 0.94
	Average		9.73	9.98	53	7.49	1.74	9.23	+ 0.75
5. (O)	" 24	60.95	9.73	9.85	60	8.65	1.94	10.59	- 0.74
	" 25	"	"	"	"	9.23	"	11.17	- 1.32
	" 26	"	"	"	"	8.77	"	10.71	- 0.86
	" 27	"	"	"	"	7.84	"	9.78	+ 0.07
	" 28	"	"	"	"	8.93	"	10.87	- 1.02
	" 29	"	"	"	"	8.63	"	10.57	- 0.73
	" 30	"	"	"	"	7.68	"	9.62	+ 0.23
	" 31	61.60	"	"	"	7.67	"	9.61	+ 0.24
	Average		9.73	9.85	60	8.43	1.94	10.36	- 0.51
6. (O)	Nov. 1		11.89	12.00	61	9.13	2.53	11.66	+ 0.34
	" 2		"	"	"	8.33	"	10.86	+ 1.14
	" 3		"	"	"	9.83	"	12.36	- 0.36
	" 4	61.40 (5. xi)	"	"	"	9.63	"	12.16	- 0.16
	Average		11.89	12.00	61	9.23	2.53	11.76	+ 0.2

the table, a quantity of stewed apples (225 g. raw fruit containing 0.11 g. N) was included in the diet. The letter (O) indicates that this fruit was omitted.

For several days prior to Sept. 30, 1920 (period 1) a bread diet containing about 7.3 g. N had been taken in order to eliminate the disturbing effect of the previous high protein dietary. For the same reason the first three days of this period and the first five days of period 2 have been excluded in calculating the average nitrogen balance.

Table V.

Diet: whole wheat bread. Subject: R.R.									
Period	Date 1920	Body weight <i>k</i>	Daily Intake			Daily Output			Balance N g.
			N bread g.	N total g.	Calories per kilo	N urine g.	N faeces g.	N total g.	
1. (A)	Sept. 30-Oct. 2	58.65 (27. ix)	5.55	5.82	48	—	—	—	—
(O)	Oct. 3-5	57.70 (6. ix)	„	5.71	49	5.62	1.47	7.09	- 1.38
2. (A)	„ 10-14	58.20 (13. x)	8.32	8.57	54	—	—	—	—
	„ 15		„	„	„	7.21	1.98	9.19	- 0.62
	„ 16		„	„	„	8.30	„	10.28	- 1.71
	„ 17	58.13	„	„	„	6.41	„	8.39	+ 0.18
		Average	8.32	8.57	54	7.31	1.98	10.29	- 0.72
3. (A)	„ 18		8.65	8.91	56	8.06	1.25	9.31	- 0.40
	„ 19		„	„	„	8.70	„	9.95	- 1.04
	„ 20		„	„	„	7.79	„	9.04	- 0.13
		Average	8.65	8.91	56	8.18	1.25	9.43	- 0.52
4. (A)	„ 21-23	57.89 (21. x)	9.73	10.00	58	8.32	1.70	10.02	- 0.02
5. (O)	„ 24		9.73	9.84	58	8.41	1.81	10.22	- 0.38
	„ 25		„	„	„	8.25	„	10.06	- 0.22
	„ 26		„	„	„	10.51	„	12.32	- 2.48
	„ 27	57.75	„	„	„	8.83	„	10.64	- 0.80
	„ 28		„	„	„	7.67	„	9.48	+ 0.36
	„ 29		„	„	„	9.74	„	11.55	- 1.71
	„ 30		„	„	„	9.50	„	11.31	- 1.47
	„ 31	57.44	„	„	„	8.54	„	10.35	- 0.51
		Average	9.73	9.84	58	8.93	1.81	10.74	- 0.90
6. (O)	Nov. 1		11.89	11.98	63	9.30	2.68	11.98	0
	„ 2		„	„	„	9.75	„	12.43	- 0.45
	„ 3		„	„	„	8.86	„	11.54	+ 0.44
	„ 4	57.70 (5. xi)	„	„	„	9.46	„	12.14	- 0.16
		Average	11.89	11.98	63	9.34	2.68	12.02	- 0.04

A marked rise in the nitrogen output occurred with both C.J.M. and R.R. at the beginning of period 5 and the coincidence of the omission of the apples that had been included in the diet during the preceding period led us to consider whether there was here any relation of cause and effect. Two possibilities suggest themselves: (1) the small amount of apple protein (0.11 g. N) might possess high value to supplement the wheat protein; (2) the alkaline

ash of the fruit would partially neutralise the acid ash of the bread and this might affect the nitrogen expenditure. Neither of the above appears adequate to explain the facts and further, the results obtained during period 4 do not agree any better with those for periods 2 and 3, in which apples were included in the diet, than with those for period 5 in which they were omitted. The increased output must therefore, like the variations which occur from day to day, be left for the present unexplained.

From the results of these experiments we have calculated the biological value of the wheat proteins by two formulae, which differ only in the assumption made with regard to the nitrogen of the faeces. Both are based on Thomas's definition of this value as the number of parts of body nitrogen spared by 100 parts of the nitrogen of the food, and, when reduced to their simplest form, can be thus expressed

$$\text{B.V.} = 100 \frac{\text{Body N spared}}{\text{Food N absorbed}} = 100 \frac{\text{Balance } [P] - \text{Balance } [M]}{\text{Intake } [P] - \text{Intake } [M]}$$

where P signifies the experiment with the protein under investigation and M the nitrogen minimum experiment.

This correction for the small amount of nitrogen in the diet of the N-minimum experiment is strictly accurate only if certain provisos hold, viz. (1) that the same amount of nitrogen in the same form, or of the same biological value, enters also into the second diet (P); (2) that the value of this nitrogen is not increased by supplementary action with the other proteins of the second diet.

In our experiments with wheat and milk proteins the first proviso is partly but not entirely satisfied. Whether or not the second is also satisfied cannot be decided. The method, however, certainly involves a less error than if the N-intake in the N-minimum experiment is altogether ignored.

When the different assumptions as to the faeces are made the two formulae become:

$$\text{I. B.V.} = 100 \frac{\text{Balance } [P] - \{\text{Intake } [M] - (\text{Urine N } [M] + \text{Faeces N } [P])\}}{\text{Intake } [P] - \text{Intake } [M]}.$$

$$\text{II. B.V.} = 100 \frac{\text{Balance } [P] - \text{Balance } [M]}{\text{Intake } [P] - (\text{Faeces N } [P] - \text{Faeces N } [M]) - \text{Intake } [M]}.$$

In I the whole of the food nitrogen is assumed to have been absorbed so that the total intake is also the real intake. In calculating the minimum expenditure corresponding with the period in question the faeces N for this period is added to the urine N $[M]$ and the intake $[M]$ subtracted from the sum. This formula corresponds with Thomas's formula B.

In II when the nitrogen of the faeces is in excess of that occurring in the N-minimum experiment this excess has been assumed to represent unabsorbed food and has been subtracted from the total intake to obtain the real intake. The minimum expenditure has been taken as the actual balance on N-minimum diet, *i.e.* Urine N $[M]$ + Faeces N $[M]$ - Intake $[M]$. This formula corresponds with Thomas's formula C except that in the latter an average

figure of 1 g. N, has been taken to represent the faeces N on a N-free diet. Both procedures have obvious disadvantages but there is very little difference in the results whichever is adopted.

A summary of the results for the separate periods with the biological values calculated from both the above formulae is given in Table VI.

Table VI.

Diet: whole wheat bread. Subject: C.J.M.						
Period	Intake			Balance N g.	Biological value	
	Total N g.	Absorbed N g.	Calories per kilo		(1)	(2)
1. (O)	5.70	4.73	44	-2.06	38.8	25.3
2. (A)	8.63	7.63	56	-1.16	36.3	27.6
3. (A)	8.97	7.79	57	-0.80	41.1	31.8
4. (A)	9.98	9.41	53	+0.75	41.0	43.2
5. (O)	9.85	9.08	60	-0.51	36.0	30.4
6. (O)	12.00	10.64	61	+0.24	40.9	33.1
Average					39.0	31.9

Diet: whole wheat bread. Subject: R.R.						
1. (O)	5.71	5.37	49	-1.38	34.2	29.8
2. (A)	8.57	7.72	54	-0.72	36.8	29.3
3. (A)	8.91	8.79	56	-0.52	28.7	27.9
4. (A)	10.00	9.43	58	-0.02	35.5	31.4
5. (O)	9.84	9.16	58	-0.90	28.0	22.5
6. (O)	11.98	10.40	63	-0.04	37.7	28.1
Average					33.5	28.2

THE BIOLOGICAL VALUE OF THE PROTEINS OF COW'S MILK.

The first experiment with nitrogen in the form of milk protein followed immediately after the second period on low nitrogen diet and was carried out in duplicate on C.J.M. and R.R. The total nitrogen intake was approximately equal to the minimum nitrogen expenditure and though equilibrium was not obtained the negative balance was not very large, from which we concluded that milk proteins would be found to possess a relatively high value. When the experiments were repeated with larger amounts of milk we were surprised to find the nitrogen balance still negative and equilibrium was only reached with diets containing over 11 g. of milk nitrogen. This result appeared so extraordinary as to lead us to suspect that it might be due to a deficiency in the energy value of the diet, although this was amply sufficient to cover our normal requirements. The unusually rapid loss in weight which occurred in some of these experiments pointed in the same direction, as did also the coincidence of the fall in the nitrogen output occurring in one period (C.J.M. 6-R.R. 3) with a pronounced rise in the temperature of the air.

A further series of experiments was therefore carried out on one of us (C.J.M. periods 7, 8, 9), the fuel value of the diet being increased to the maximum that could be consumed. In the first of these, nitrogen equilibrium

was practically attained with a diet containing 6.84 g. N and furnishing 57 calories per kilo. After an interval of one day, on which the basal ration together with a very little milk (3.0 g. N) was consumed, period 8 was begun. The diet contained 5.28 g. N and furnished 55 calories per kilo, but the effects of the continued excessive diet made themselves unpleasantly obvious in the form of a bilious attack, which threatened to terminate the experiment. By reducing the amount of carbohydrate consumed, so that the fuel value fell to 36 calories per kilo it was however just possible to carry on and after two days the condition was so far improved that the full diet was resumed. The nitrogen intake was not altered at all but the effect of the reduced diet was very marked in the increased nitrogen output, which persisted for several days after the calories were again increased. The period was extended for six days after the output had reached a fairly constant level and only the results for these days have been considered in calculating the average output. A decided though small negative balance occurred.

Table VII.

Milk. C.J.M.

Foodstuff	Nitrogen per 100 g.	Calories per 100 g.	Diet during period 9 20-25. viii. 21		
			Weight g.	Nitrogen g.	Calories
Milk	0.508	65	830	4.216	539
Starch	0.027	360	280	0.076	1008
Lactose	0.013	370	210	0.027	777
Honey	0.023	327	250	0.057	818
Margarine (rendered)	0.010	900	18	0.002	162
Sucrose	—	395	10	—	40
Tea	0.008	—	750 cc.	0.060	—
Agar-agar	0.242	—	6	0.014	—
Salts	—	—	4	—	—
			Total	4.452	3344
			Excluding tea N	4.392	54 per kilo

In period 9 the quantity of milk was again reduced but the high calorie value of the diet was maintained. The usual daily game of tennis was discontinued and no exercise was taken so that the excess of energy supplied was even greater than before. The weather also was very hot. The average of the last six days of this period showed a considerable negative balance and the biological value calculated from this agrees fairly well with that obtained from the results of periods 7 and 8 and also of period 2 (C.J.M.). This point is interesting because the calorie value of the diet in period 2 was lower than in any other, only 44 per kilo. The composition of the diet during period 9 (C.J.M.) is given in Table VII and the results of the experiments are set out in Tables VIII (R.R.) and IX (C.J.M.), while Table X is a summary of these showing the biological values calculated from the two formulae. It is obvious from the amounts of nitrogen in the faeces that the milk proteins were very completely absorbed so that formula 1 probably gives the closest approximation to the truth in this case.

Table VIII.

Diet: milk. Subject: R.R.

Period	Date 1920	Body weight <i>k</i>	Daily Intake			Daily Output			Balance N g.
			N milk g.	N total g.	Calories per kilo	N urine g.	N faeces g.	N total g.	
“N-free” 1.	Dec. 5	57.75	0	0.27	44	2.01	1.13	3.14	-2.87
	„ 6		2.73	2.98	48	2.49	0.66	3.15	-0.17
	„ 7		2.69	2.94	„	2.95	„	3.61	-0.67
	„ 8		2.76	3.09	„	3.22	„	3.88	-0.79
	„ 9		2.59	2.91	„	2.86	„	3.52	-0.61
	„ 10	57.50	2.67	2.97	„	2.83	„	3.49	-0.52
		(11. xii)							
	Average of last 4 days		2.68	2.98	48	2.97	0.66	3.63	-0.65
2.	1921								
	May 28	59.65	4.10	4.30	40	7.12	—	—	—
	„ 29		6.14	6.29	48	5.97	1.18	7.15	-0.86
	„ 30		„	„	„	5.91	„	7.09	-0.80
	„ 31		„	„	„	6.34	„	7.52	-1.23
	June 1		„	„	„	7.09	„	8.27	-1.98
	„ 2		„	„	„	6.55	„	7.73	-1.44
	„ 3	57.90	„	„	„	6.21	„	7.39	-1.10
		(4. vi)							
	Average of last 4 days		6.14	6.29	48	6.55	1.18	7.73	-1.44
3.	June 16		Mixed diet			12.29	—	—	—
	„ 17	59.60	13.61	13.69	47	13.48	1.37	14.85	-1.16
	„ 18		„	„	„	13.13	„	14.50	-0.81
	„ 19		„	„	„	12.29	„	13.66	+0.03
	„ 20		„	„	„	13.76	„	15.13	-1.44
	„ 21		„	„	„	12.39	„	13.76	-0.07
	„ 22		„	„	„	12.60	„	13.97	-0.28
	„ 23	58.50	„	„	„	11.10	„	12.47	+1.22
	„ 24		„	„	„	11.19	„	12.56	+1.13
		Average of last 5 days		13.61	13.69	47	12.21	1.37	13.58

Table IX.

Diet: milk. Subject: C.J.M.

Period	Date 1920	Body weight <i>k</i>	Daily Intake			Daily Output			Balance N g.
			N milk g.	N total g.	Calories per kilo	N urine g.	N faeces g.	N total g.	
“N-free” 1.	Dec. 5	60.45	0	0.33	48	2.13	1.17	3.30	-2.97
	„ 6		3.15	3.37	49	2.42	1.29	3.71	-0.34
	„ 7		„	3.36	„	2.57	„	3.86	-0.50
	„ 8		„	3.42	„	3.01	„	4.30	-0.88
	„ 9		„	3.40	„	3.12	„	4.41	-1.01
	„ 10	59.30 (11. xii)	„	3.45	„	3.22	„	4.51	-1.06
	Average of last 3 days		3.15	3.42	49	3.12	1.29	4.41	-0.99
2.	1921								
	April 30	62.60	4.02	4.17	44	4.74	1.22	5.97	-1.80
	May 1		„	„	„	4.58	„	5.80	-1.63
	„ 2		„	„	„	4.09	„	5.32	-1.15
	„ 3		„	„	„	4.25	„	5.47	-1.30
	„ 4	62.20	„	„	„	4.53	„	5.75	-1.58
	Average of last 4 days		4.02	4.17	44	4.36	1.22	5.58	-1.41
3.	May 10		Mixed diet			8.00	—	—	—
	„ 11	62.20	6.20	6.35	48	6.13	1.18	7.31	-0.96
	„ 12		„	„	„	5.71	„	6.89	-0.54
	„ 13		„	„	„	5.26	„	6.44	-0.09
	„ 14		„	„	„	5.80	„	6.98	-0.63
	„ 15		„	„	„	6.08	„	7.26	-0.91
	„ 16		„	„	„	6.07	„	7.25	-0.90
	„ 17	62.15 (18. v)	„	„	„	6.21	„	7.39	-1.04
	Average of last 4 days		6.20	6.35	48	6.04	1.18	7.22	-0.87

Table IX (*continued*)

Diet: milk. Subject: C.J.M.									
Period	Date 1921	Body weight <i>k</i>	Daily Intake			Daily Output			Balance N g.
			N milk g.	N total g.	Calories per kilo	N urine g.	N faeces g.	N total g.	
"N-free" 4.	May 27		—	—	—	8.38	—	—	—
	" 28	62.40	7.63	7.78	47	7.05	0.96	8.01	-0.23
	" 29		"	"	"	7.11	"	8.07	-0.29
	" 30		"	"	"	7.55	"	8.51	-0.73
5.	" 31		8.62	8.77	47	7.48	0.98	8.46	+0.31
	June 1		"	"	"	8.58	"	9.56	-0.79
	" 2	61.30	"	"	"	8.41	"	9.39	-0.62
	" 3		"	"	"	8.67	"	9.65	-0.88
Average of last 3 days			8.62	8.77	47	8.55	0.98	9.53	-0.76
6.	June 16		Mixed diet			10.12	—	—	—
	" 17	61.80	11.32	11.44	46	9.71	1.45	11.16	+0.28
	" 18		"	"	"	10.46	"	11.91	-0.47
	" 19		"	"	"	10.21	"	11.66	-0.22
	" 20		"	"	"	10.19	"	11.64	-0.20
	" 21		"	"	"	10.82	"	12.27	-0.83
	" 22		"	"	"	8.09	"	9.54	+1.90
	" 23		"	"	"	9.60	"	11.05	+0.39
	" 24		"	"	"	9.71	"	11.16	+0.28
	" 25	61.70 (26. vi)	"	"	"	10.28	"	11.73	-0.29
Average of last 6 days			11.32	11.44	46	9.78	1.45	11.23	+0.21
7.	July 2		Mixed diet			6.33	—	—	—
	" 3	61.10	6.65	6.84	57	5.66	1.28	6.94	-0.10
	" 4		"	"	"	5.55	"	6.83	+0.01
	" 5		"	"	"	5.35	"	6.63	+0.21
	" 6		"	"	"	5.70	"	6.98	-0.14
	" 7		"	"	"	6.35	"	7.63	-0.79
	" 8	61.80	"	"	"	5.04	"	6.32	+0.52
Average of last 4 days			6.65	6.84	57	5.61	1.28	6.89	-0.05
8.	July 9	62.10	—	3.00	54	5.16	—	—	—
	" 10		5.16	5.28	55	4.54	1.09	5.63	-0.35
	" 11		"	"	36	3.99	"	5.08	+0.20
	" 12	61.00	"	"	36	5.57	"	6.66	-1.38
	" 13	60.80	"	"	53	5.28	"	6.37	-1.09
	" 14	61.10	"	"	57	5.29	"	6.38	-1.10
	" 15	61.30	"	"	55	4.71	"	5.80	-0.52
	" 16	61.60	"	"	54	4.63	"	5.72	-0.44
	" 17	61.90	"	"	53	4.83	"	5.92	-0.64
	" 18	61.85	"	"	52	4.06	"	5.15	+0.13
	" 19	61.85	5.04	5.18	52	4.65	"	5.74	-0.56
	" 20	62.00	"	"	52	4.24	"	5.33	-0.15
Average of last 6 days			5.12	5.25	53	4.52	1.09	5.61	-0.36
9.	Aug. 16	61.60	4.24	4.58	53	15.79	—	—	—
	" 17	61.85	"	"	"	10.78	—	—	—
	" 18	61.85	"	"	"	6.46	—	—	—
	" 19	61.90	"	"	"	5.83	—	—	—
	" 20		"	4.39	"	4.69	1.00	5.69	-1.30
	" 21	61.80	"	"	"	4.00	"	5.00	-0.61
	" 22	61.80	"	"	"	4.10	"	5.10	-0.71
	" 23	61.60	"	"	"	5.04	"	6.04	-1.65
	" 24	61.60	"	"	58	4.78	"	5.78	-1.39
	" 25	61.75	"	"	55	4.72	"	5.72	-1.33
Average of last 6 days			4.24	4.39	55	4.56	1.00	5.56	-1.17

Table X.

Diet: milk. Subject: C.J.M.

Period	Intake			Balance N g.	Biological value	
	Total N g.	Absorbed N g.	Calories per kilo		(1)	(2)
1.	3.42	3.30	49	-0.99	74.7	73.6
2.	4.17	4.12	44	-1.41	47.3	46.6
3.	6.35	6.34	48	-0.87	38.4	38.3
5.	8.77	8.77	47	-0.76	26.3	28.6
6.	11.44	11.16	46	+0.21	33.0	31.2
7.	6.84	6.73	57	-0.05	49.7	48.8
8.	5.25	5.25	53	-0.36	55.6	57.2
9.	4.39	4.39	55	-1.17	45.2	49.4
Average for last 3 periods					50.2	51.8

Diet: milk. Subject: R.R.

1.	2.98	2.98	48	-0.65	66.0	83.6
2.	6.29	6.24	48	-1.44	25.0	24.4
3.	13.69	13.45	47	+0.11	24.2	22.9

Table XI. *Basal metabolism of C.J.M. on normal diet and on carbohydrate and fat (= 55 cal. per kilo) with 4.4 g. milk N.*

Date	Diet during previous 24 hrs.	Oxygen consumed per min. cc.	R.Q.	Calories per 24 hrs.
Aug. 10	Normal	225.6	0.711	1557
" 12	"	236.3	0.928	1699
" 13	"	227.1	0.684	1566
" 14	"	220.9	0.820	1557
				Average 1595
" 20	Milk	211.5	0.974	1529
" 21	(4.39 g. N, 55 cal. per kilo)	178.0	1.159	1359
" 22	"	197.3	1.093	1478
" 23	"	185.7	1.152	1415
" 24	"	195.0	1.044	1440
" 25	"	196.1	1.055	1453
" 26	"	210.4	1.017	1540
				Average 1459
" 27	Normal	221.9	0.915	1590
" 28	"	216.8	0.889	1550
" 31	"	228.0	0.811	1604
				Average 1581

During period 9 the subject's basal metabolism was determined on waking and the results were compared with similar determinations carried out during previous and succeeding periods when the diet was normal. These results are set out in Table XI and show a decrease of about 8 % in the basal metabolism on the milk diet.

DISCUSSION OF RESULTS.

THE MINIMUM NITROGEN EXPENDITURE.

During the second experiment to determine our nitrogen minimum the output of nitrogen in the urine fell to an amount equal to 0.035 g. per kilo body weight in the case of C.J.M. and 0.034 g. per kilo for R.R. The average amounts for the last three days of this period were slightly above the minima, being 0.038 g. and 0.035 g. respectively. If the nitrogen of the faeces is included the average output for the same period was 0.057 g. per kilo for C.J.M., and 0.055 g. for R.R. The nitrogen intake amounted to 0.005 g. per kilo.

These figures are somewhat lower than most of those recorded by other workers [Landergren 1903; Folin, 1905; Kinberg, 1911; Graham and Poulton, 1912], but this may be explained by the fact that their diets unavoidably contained more nitrogen than ours. Karl Thomas [1910] determined his minimum expenditure on a purely carbohydrate diet in seven experiments carried out over a period of two and a half years and found that this minimum fell from experiment to experiment, the final amount for the output in the urine alone being 2.2 g. or 0.029 g. per kilo, while that for urine and faeces combined was 2.9 g. or 0.039 g. per kilo. At this period Thomas weighed 75 kilos and had put on a good deal of body fat. In some of his earlier experiments, however, it seems probable that the minimum output was never reached as the diet was continued for too short a period.

In McCollum's [1911] experiments on pigs, which were fed on a diet of starch, a salt mixture and water, the minimum nitrogen output fell to a level corresponding closely with that reached by us. Thus a pig weighing 68.4 kilos excreted 0.039 g. N per kilo in the urine. For smaller animals the output per kilo was somewhat greater.

The determination of the nitrogen in urine and faeces does not, of course, give a complete account of the loss of nitrogen from the body. To these must be added the loss through hair, beard and nails, through loss of epidermis and in sweat. Except for the last named these losses are all very small in amount but the loss through the sweat may be considerable. Benedict [1906] has shown that a resting man may excrete 0.071 g. N per day in this way while with moderate work the loss may amount to 0.13 g. N per hour. McCollum considers that the nitrogen of the faeces should also be classed with these as representing losses that may be termed accidental in character and that the nitrogen of the urine alone is to be taken as representing the essential tissue metabolism of the body.

The amount of nitrogen excreted in the urine on the successive days of the experiments in which the diet was nearly free from nitrogen, is plotted in Figs. 2, 3, and 5. The amount diminishes in a fairly regular manner, at first quickly and then more slowly until the minimum is reached. The points lie on or near the graph of a simple logarithmic equation

$$\log (y - \lambda) = a - kx$$

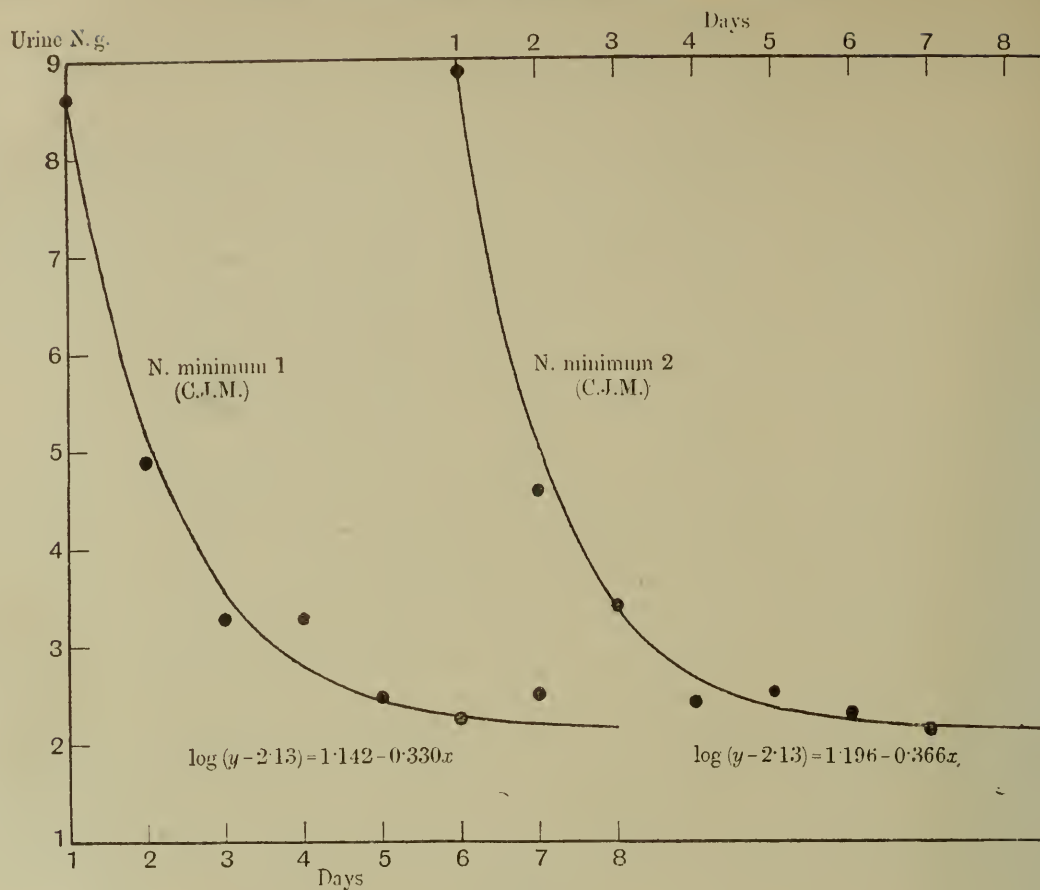


Fig. 2.

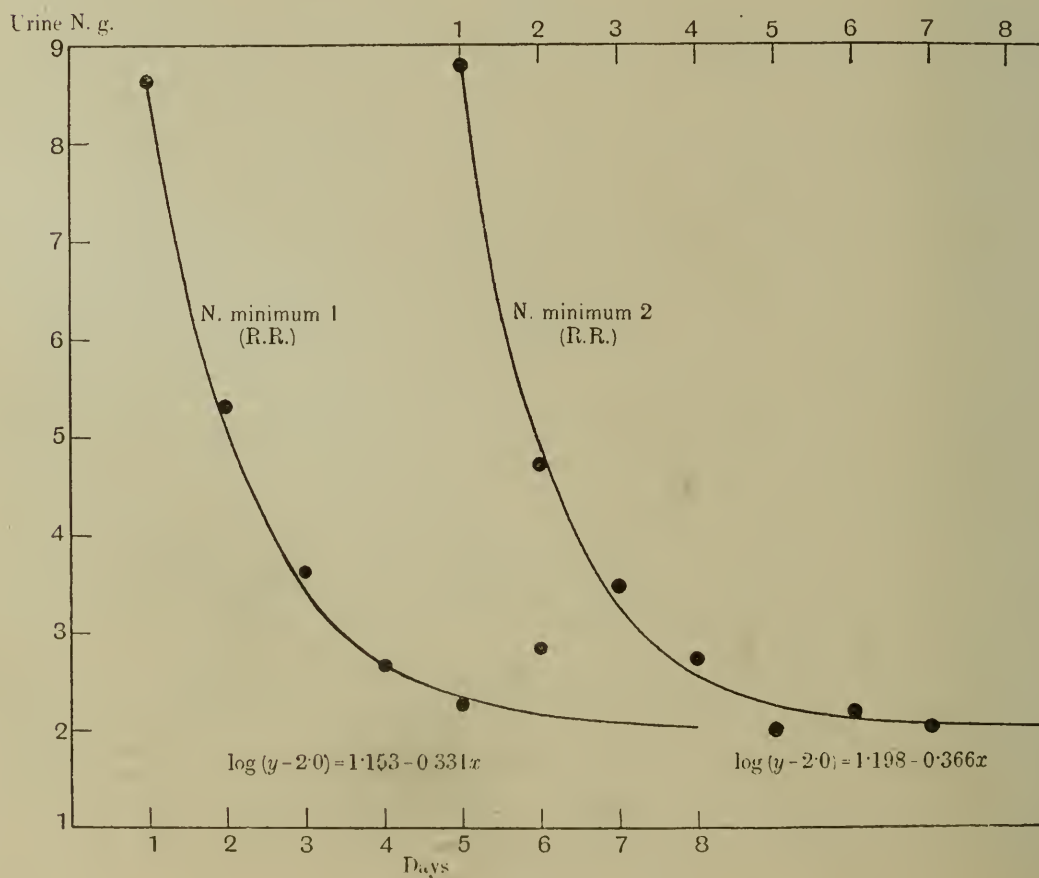


Fig. 3.

in which x is the number of days, y is the nitrogen output in the urine, and λ the minimum value of y . A closer agreement is obtained if λ is given a value slightly lower than the minimum actually reached.

Thus in Fig. 3 the curve R.R. 1 is drawn from the equation

$$\log (y - 2.0) = 1.153 - 0.331x$$

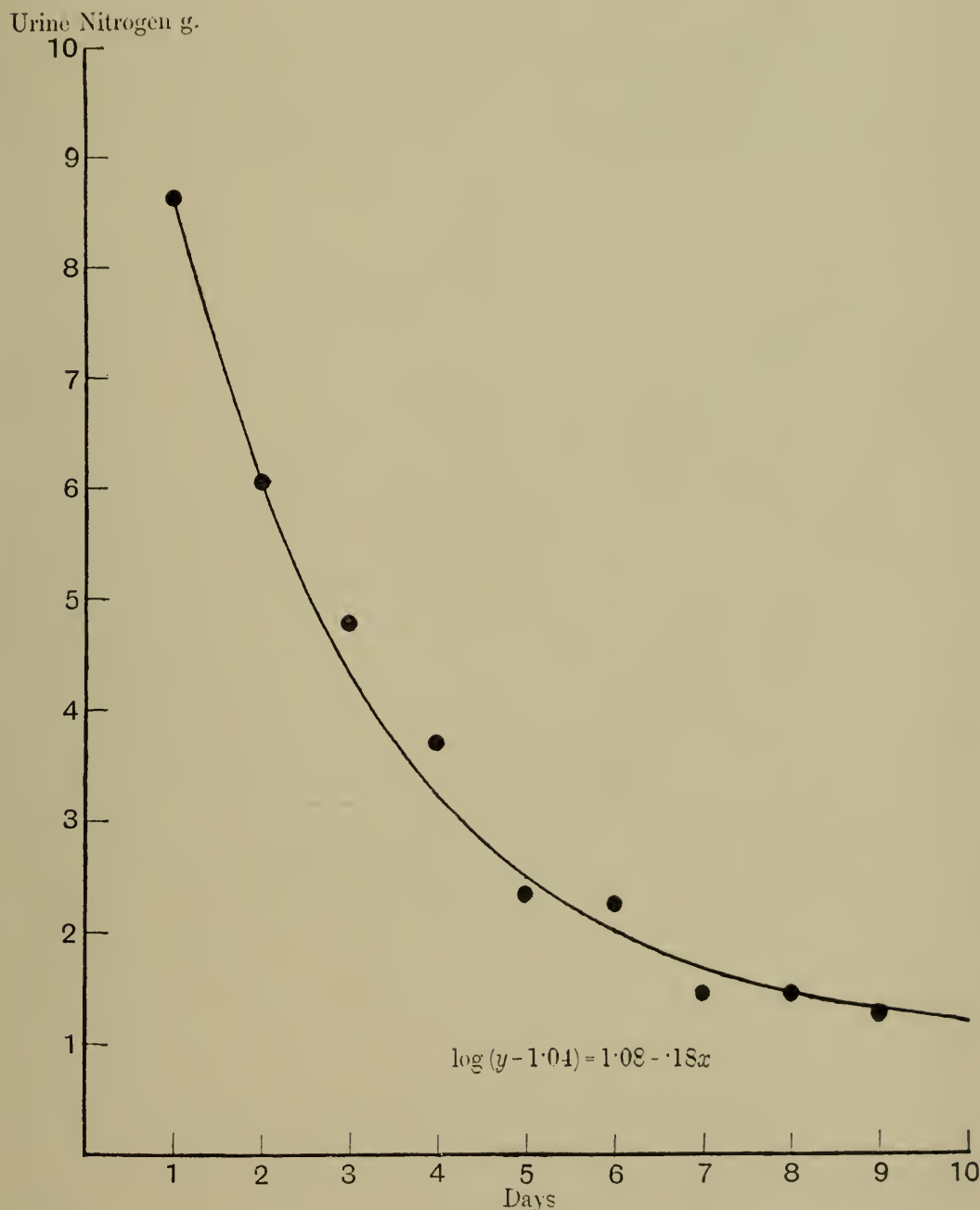


Fig. 4. E. V. McCollum's experiment on a pig. Daily output of nitrogen in urine on starch diet after ingestion of zein.

and fits the points reasonably well, but a still closer approximation is obtained with the equation $\log (y - 1.73) = 1.1209 - 0.2832x$

the agreement between the observed and calculated values of y being extraordinarily good for all points except the last.

Day of experiment	y calculated from equation $\log (y - 1.73) = 1.1209 - 0.2832x$	y obtained
1	8.61	8.64
2	5.31	5.31
3	3.60	3.63
4	2.70	2.66
5	2.24	2.25
6	1.99	2.82
20	1.73	—

A question is thus raised: Does this amount 1.73 g. represent the real minimum expenditure, which, from some cause was not realised in either experiment? At present this must be left unanswered, but further experiments may give some information on the point.

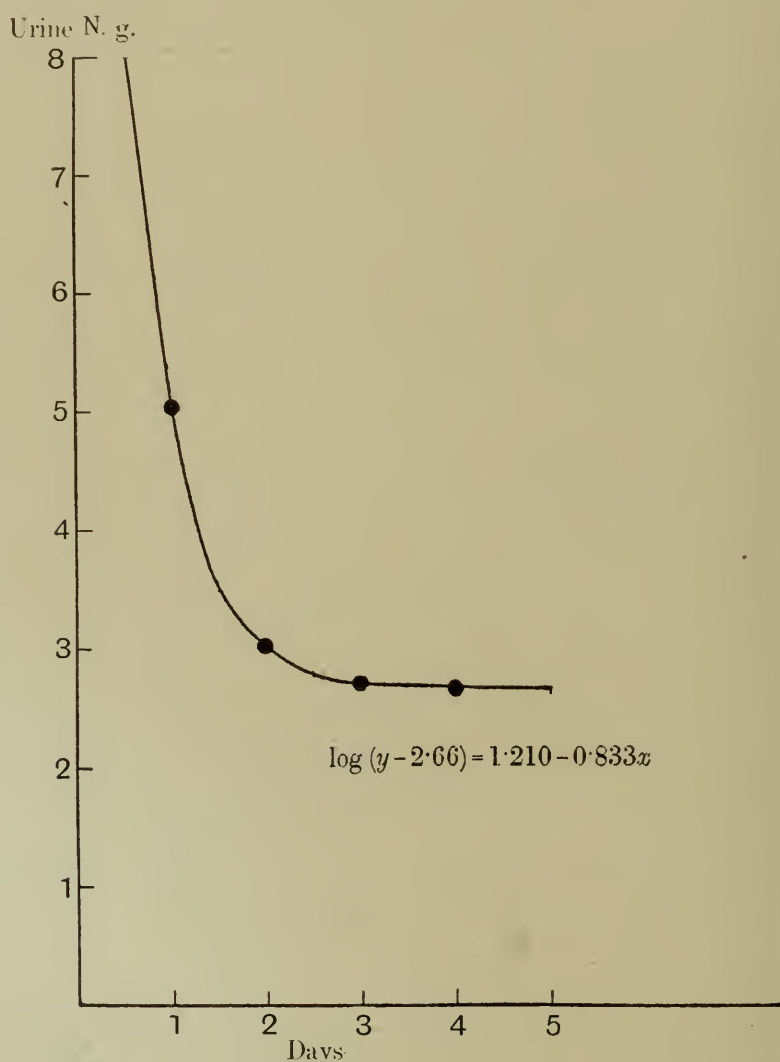


Fig. 5. E. V. McCollum's experiment on a pig. Daily output of nitrogen in urine on starch diet after ingestion of urea.

The difference between experiments 1 and 2 lies only in the amount of total fluid taken which was about 2000 cc. daily in the first and 4000 cc. in the second. This makes but slight difference in the value of k , that is in the rate at which the nitrogen output falls. Kinberg [1911] has previously drawn attention to this regularity but has not attempted to deduce any mathematical

expression from his results. Thomas [1910] also recognised that on a protein-free diet the nitrogen of the "Vorratseiwiss" leaves the body with varying rapidity according to the amount present, but was unable to find any exact relationship either in his own results or in those of Landergren. He calculated the amount of "Vorratseiwiss" excreted, by subtracting the minimum output ("Abnutzungsquota") from the urine nitrogen, but the value of this minimum was taken from experiments of four days' duration and was probably too high. Landergren's results show a fair agreement with the graph of an equation of the type given above if λ is taken as 2.5 instead of 3.0. Thomas's results are more irregular. In considering the agreement of the results of such experiments

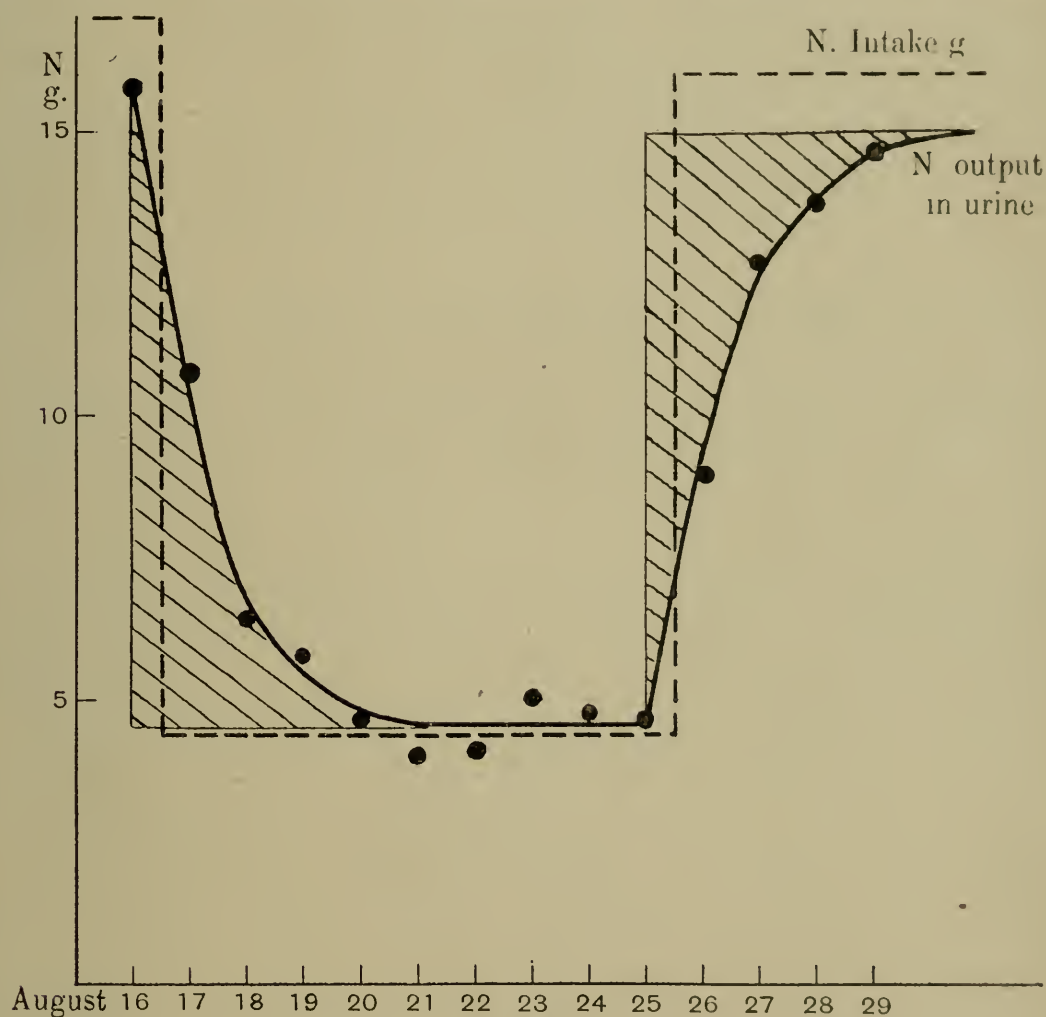


Fig. 6.

with those calculated from these equations, the tendency of the nitrogen output to oscillate even when the nitrogen intake is constant must be borne in mind. Such oscillations are present in most of the experiments considered, but do not alter the general character of the curve. In McCollum's experiments on pigs a similar regularity also appears as may be seen from Figs. 4 and 5. Fig. 4 shows the nitrogen output on a starch diet after a diet containing zein and Fig. 5 shows the output after the ingestion of urea. The curves are of the same type but " k ," i.e. the rate at which the output falls, is very much

greater after urea than after zein. This point is of interest as it seems to indicate that the store of nitrogen which exists in the body after a protein diet, and which is rapidly given up on a diet free from nitrogen is not present entirely in the form of urea. As to the form in which this nitrogen occurs, very little can be definitely stated. It may be as resynthesised protein (Vor-ratseiweiss), or as amino acids adsorbed in the tissues, or compounds of intermediate complexity. It is certainly present partly as urea and other end products of metabolism. If our conclusions as to the rate at which this storage nitrogen is excreted are correct the interpretation is, that the amount removed from the body on any day is proportional to the amount still present. This might hold whether the reaction involved was the hydrolysis of protein, the deamination of amino acids or simply the washing out from the tissues of the end products of nitrogen metabolism.

On reversing the process, and after a minimal N-intake for ten days, suddenly increasing the nitrogenous food consumed, nitrogen at first remains in the body and equilibrium between intake and output does not occur for several days. Fig. 6 is a graph of the results of an experiment designed to show this. The broken line represents intake of N, the solid line output. The shaded area on the left hand represents the stored N gradually removed on dropping the intake from 17 g. to 4.4 g. and the right-hand area the amount again stored on resuming a diet containing 16 g. N. The curves are reciprocal and the two shaded areas approximately correspond.

THE NATURE OF THE MINIMUM NITROGEN REQUIREMENTS OF THE BODY.

The low level to which the nitrogen output falls on a protein free diet is evidence of the smallness of the body's daily requirements in this respect. We know from Folin's [1905] researches that the reduction in the nitrogen on such a diet occurs mainly at the expense of the urea fraction, the ammonia and uric acid being reduced to a relatively much smaller extent while the creatinine remains constant. These facts led Folin to conclude that protein metabolism is of two types, (1) "tissue" or "endogenous," which tends to be constant, and is represented largely by such products as creatinine, neutral sulphur, and to a less extent by uric acid, (2) "exogenous" which varies with the amount of protein consumed and is represented chiefly by urea. The nitrogen required for processes of the first type is alone essential, but Folin recognised that equilibrium at this low level may not be possible since a certain amount of protein may always fall prey to the exogenous metabolism. The distribution of the nitrogenous constituents of the urine was determined during our second minimum experiment and the milk diet immediately succeeding this.

The results [Robison, 1922, 2] are similar to those of Folin. The minimum nitrogen output was lower than any recorded by him, and the percentage of urea was correspondingly reduced, the minimum figure being 37.2 %, while the sum of the urea and ammonia amounted to 54.8 % of the total nitrogen.

The multifarious transactions involved in endogenous metabolism are not likely to be conducted with perfect economy. When much protein is hydrolysed and the products mobilised and used for the synthesis of proteins of another composition such as those of the blood or for the manufacture of thyroxin or adrenaline it is unlikely that the whole balance of unwanted amino acids escapes deamination and conversion. Leakage of this kind may account for no inconsiderable fraction of the minimum nitrogen expenditure and it is perhaps in this direction that, by adaptation, the body may effect some saving. The experiments of Thomas and Hindhede would appear to show that this does in fact occur.

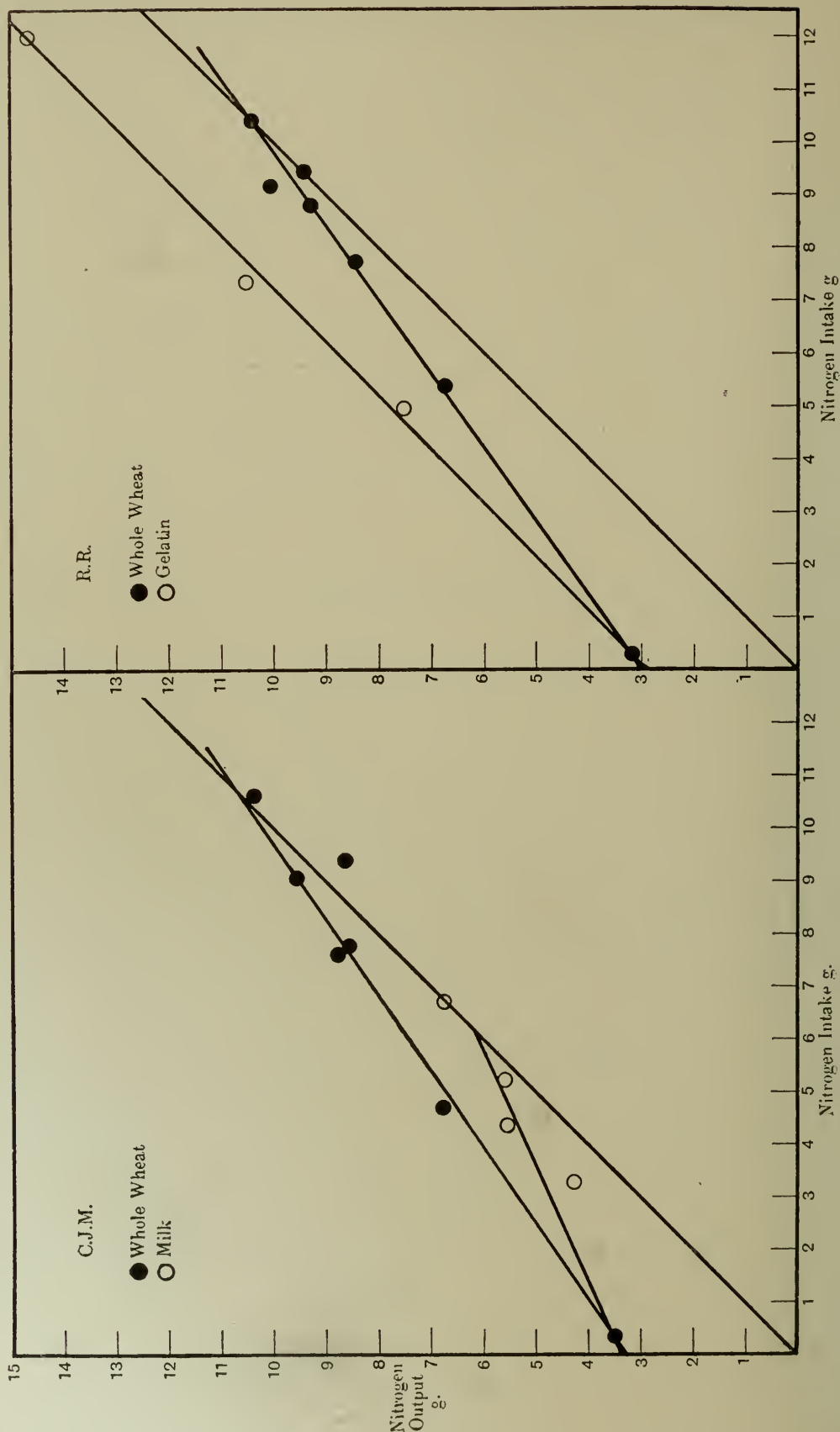
The amount of carbohydrate eaten may also influence the degree of this waste since the process of deamination is reversible and will be affected by the rapidity with which the non-nitrogenous products, hydroxy or ketonic acids, are removed by oxidation or conversion into carbohydrate. It is probable that an excess of carbohydrate in the blood would retard either action and in consequence deamination.

THE QUESTION OF UNIFORMITY OF THE BIOLOGICAL VALUE.

We have seen that the validity of the method adopted by Thomas for the determination of the biological values of proteins depends in the first place on the uniformity of this value when varying amounts of the same protein are consumed. The investigation of this question was one of the objects of our experiments and the results must now be considered from this point of view. The three proteins so far studied were chosen on account of the wide difference in the values attributed to them. The biological values of milk and wheat proteins as given by Thomas are 99.71 and 39.56 respectively, while gelatin has long been known to be deficient in several essential amino acids, so that no amount, however great, can completely satisfy the body's nitrogen requirements.

The results of the experiments described in this paper together with those on gelatin, previously recorded by one of us [Robison, 1922, 1] are plotted in Figs. 7 and 8, in which the values of the nitrogen intake for different periods are the abscissae and the corresponding amounts for the total output in urine and faeces are the ordinates. For this purpose the nitrogen of the faeces in excess of the amount on the "protein-free" diet has been taken as representing unabsorbed food and has been subtracted from both intake and output.

In the case of whole wheat proteins, the results are strikingly similar with both individuals and the points obviously lie on or close to a straight line passing through the points representing the minimum expenditure on the low nitrogen diet. The agreement is remarkably good considering the errors inseparable from such experiments. The results for periods 4 and 5 show some discrepancy but these periods were continuous and should probably be considered as a whole, the only difference in the diets being the inclusion of



a small quantity of apples in the former and its omission from the latter. The average for the two periods gives a point lying close to the line. The uniformity of the biological value is therefore satisfactorily proved for the proteins of whole wheat.

In the case of gelatin also the points fit a straight line reasonably well, but if this line passes through the point representing the nitrogen minimum (as drawn in the figure), it will at some distant point intersect the "equilibrium line" (at 45° to the axis), *i.e.* nitrogenous equilibrium will be attained at this point, which we believe to be impossible. On the other hand if it is drawn parallel to the "equilibrium line" it will cut the axis of y at a point slightly below the observed minimum. We must conclude, therefore, either that the line is slightly curved near the minimum or that the observed value of the latter is somewhat higher than the real one. If the capacity of the gelatin is limited to the reduction of "leakage" these two alternatives have practically the same significance.

Milk presents a more difficult case. Only the results for those periods in which a diet of abundant fuel value was taken (C.J.M. 1, 7, 8, 9) have been plotted but even these do not agree well with any straight line or other regular curve. The most that can be said is that in view of the possible errors in such experiments where the intake is small, the results are not inconsistent with the uniformity of the biological value of this protein.

From the consideration of these three cases we may conclude that the general assumption of this principle made by Thomas, occasioned no serious errors in his conclusions. Caution must be used in extending this principle to all cases without investigation and reliance should not be placed upon results of experiments in which the negative balance is large.

THE BIOLOGICAL VALUES OF THE PROTEINS OF WHOLE WHEAT AND MILK.

Whole Wheat.

The biological values calculated from the different periods of our experiments with whole wheat proteins agree very well amongst themselves and give an average of 35 for those obtained on C.J.M. and 31 for those obtained on R.R. Thomas's value is somewhat higher, 39.56. The latter figure was based on the result of two experiments each of three days' duration and one of four days. During none of these experiments was the nitrogen intake constant; in one, the amounts for the separate days were 4.0 g., 7.3 g., 9.0 g. respectively. The minimum requirements corresponding with these three periods were taken as 4.63 g., 3.991 g. and 3.316 g. N (urine only), these amounts being determined in experiments of four and three days' duration. In the first of these the nitrogen output on the four days was 18.32 g., 10.17 g., 7.39 g., 4.63 g. but there is no evidence that the last figure represented Thomas's minimum requirements. The biological value of wheat proteins was calculated from each separate

day's balance and out of these widely varying results those for certain days were selected in a somewhat arbitrary fashion. The experimental results of his third period, in which the intake was nearly constant, indicate a similar biological value for wheat proteins to that found by ourselves.

The result Hindhede [1913, 1] obtained upon F. Madsen with white bread, namely a positive balance of about 1 g. per day over a 28 day experiment on 13.73 g. N was certainly not a minimum quantity and the amount necessary for N equilibrium may be considerably less. His further experiments [1914] were made with rye bread ("Schwarzbrot") and the diets contained considerable amounts of fruit or vegetables, which accounted for 0.5 g. to 1.9 g. N. These experiments were carried out in duplicate on F. and H. Madsen and were continued for four months so that they possess great value. In the final period of six days the fruit was omitted and positive balances were obtained on 13.52 g. (F.M.) and 10.68 g. (H.M.) bread nitrogen respectively. By subtracting the whole of the nitrogen in the faeces Hindhede calculates that the amounts absorbed were 8.49 g. and 6.28 g. respectively. We do not agree with this method of treating the faecal nitrogen and cannot accept his conclusion that bread proteins possess equal value with those of potatoes, of meat and of the body; but there is no doubt that in his experiments, equilibrium was obtained on a lower intake of nitrogen in the form of rye bread (and still lower when supplemented by fruit) than our minimum for whole wheat. This is confirmed by the experiments of Neumann [1919], who, by long continued diet of very high calorie value, was ultimately able to retain nearly 3 g. N daily with an intake of 9.9 g. N in the form of rye proteins. Neumann's experiment was upon himself, and was in every way unexceptionable, but it may perhaps be significant that it was preceded by a prolonged period of semistarvation during which he was investigating the German civilian ration of 1916-17. This is a further indication that, apart from the influence of loss of body weight, the organism can gradually accommodate itself to a lower ration of nitrogen, perhaps by the exercise of greater economy.

Abderhalden's [1915] observations on Röse, although as pointed out earlier in this paper, not susceptible of the interpretation he places upon them, do indicate that the latter could get into equilibrium with about a gram less N in the form of white bread than we could with bread made from the whole grain. Röse's nitrogen expenditure on a nitrogen-free diet was not ascertained so we cannot estimate the biological value for this diet.

The recent observations of Rubner [1919] upon the proteins of white flour are interesting in relation to our own, because they show, we think, that from the point of view of biological value, the proteins of the endosperm are equal, if not superior to, those of the whole seed.

In most of Sherman's experiments on the value of cereal proteins these are supplemented by a certain amount of milk and the results are not directly comparable with ours, but in some experiments on white bread [1920] the diet consumed would not appear to be greatly different from that taken by

us and we are therefore the less able to explain the difference between his results and ours. Sherman's subject, a man weighing 80 kilos, attained equilibrium on a diet containing 6.0 g. N over 95 % of which was derived from white bread and the remainder from apples and butter. The energy value was only 34 calories per kilo. The bread was purchased from a bakery and probably contained a small amount of milk but how much was not known.

Milk.

The results of our experiments with milk proteins do not agree so closely as those for the whole wheat. The very low values calculated from the results of periods 3, 5, 6 (C.J.M.) and 2, 3, (R.R.) are not easy to explain. That they are in some way due to the lower calorie value of the diet seems clear but this was in no case below that of our normal diet and amply covered our energy requirements. The high values obtained with both subjects in period 1 are perhaps connected with the previous nitrogen starvation and a consequent increase in the economy with which the body may deal with the protein supplied to it.

If we consider only periods 7, 8, 9 (C.J.M.) in which the conditions were the same and the energy supply abundant the biological value for milk proteins is equal to 51 %. This value is only half of that found by Thomas, but the criticisms we have made in discussing his experiments with bread apply with still greater force in this case. His value (100 %) was calculated from the nitrogen balance on a single day of an experiment lasting only two days on which the intake was 6.24 g. and 7.28 g. respectively. The minimum requirements were taken as 3.99 g. which is probably much too high. If the value of milk protein were as high as Thomas makes out it is difficult to see how he could explain the large negative balances occurring in his experiment with "Frauenmilch" (cow's milk with extra sugar and cream). During the first two days of this experiment the fuel value of his diet was obviously too low, but in the last three days it was equal to 40-45 calories per kilo, the N-intake being 15.3 g.-17.3 g. yet the negative balance was never less than 1.0 g. The results of this experiment appear to agree with our experience, and to suggest that a high intake of milk nitrogen tends to result in increased expenditure of body nitrogen, unless the fuel value of the diet is raised very much above the normal. So far as we are aware the value of milk protein has not been the subject of any other investigation on man.

CONCLUSIONS.

From our observations upon ourselves we conclude:

- (1) That our minimum nitrogen expenditure by the urine is somewhat less than 0.038 g. and 0.035 g. per kilo in C.J.M. and R.R. respectively.
- (2) That on taking a diet of carbohydrate and fat of adequate calorie-value the nitrogen excreted in the urine falls in a regular and orderly manner,

capable of simple mathematical expression, approaching a minimum in five to seven days. On resuming an ordinary nitrogenous diet the reciprocal phenomenon occurs.

(3) Bearing in mind the considerable experimental errors, the ratio $\frac{\text{Body N saved}}{\text{Food N absorbed}}$ appears to remain constant, whatever amount of nitrogen is taken in the form of whole wheat bread, until equilibrium is reached.

(4) In the case of milk the experimental errors are proportionately greater and the most we can say is that this ratio may remain constant.

(5) In the case of gelatin the ratio certainly does not remain constant and there is no indication that the amount of body nitrogen saved increases beyond that effected by the smallest quantity of gelatin fed.

(6) The application of Thomas's method of determining biological values is justified in the case of bread, doubtful with milk and impossible with gelatin.

(7) Until Thomas's procedure has been ascertained to be justifiable for the particular proteins concerned, the ratio $\frac{\text{Body N saved}}{\text{Food N absorbed}}$ should be determined close to, but below, the point of equilibrium.

(8) The mean biological value of the nitrogen contained in the whole wheat grain as determined by six experiments on each of two adults was 35 % (C.J.M.) and 31 % (R.R.).

(9) The mean biological value of the nitrogen in cow's milk, derived from three experiments upon C.J.M. in which an excess of calories (55 per kilo) was taken, was 51 %.

(10) Biological values arrived at from experiments of comparatively short duration, however well justified, have a limited significance.

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XVI. THE VALUE OF GELATIN IN RELATION TO THE NITROGEN REQUIREMENTS OF MAN.

BY ROBERT ROBISON.

From the Lister Institute.

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THE story of the earliest attempts to discover the value of gelatin as a food-stuff has been told by Carl Voit [1872] in the introduction to his paper on this subject. It commences in 1682 when Dionys Papin prepared gelatin from bones by means of his digester, and from the gelatin made soup with which he fed the poor. Such attempts were zealously renewed during the French Revolution by Cadet de Vaux, d'Arcet and others, and were supported by the Government, who issued official instructions extolling the nourishing properties of gelatin soup above those of beef tea. The approbation of the Institute of France and of the Academy of Medicine was also forthcoming, but in spite of d'Arcet's attempts to improve the flavour of the soup with spices, it did not meet with very great approval from the poor, who were expected to consume it.

Gannal, a manufacturer of gelatin, fed himself and his family on gelatin, with and without bread, for some weeks until compelled to desist owing to the unsupportable nausea caused by the diet. The effects on the health of these people led him to conclude that gelatin is not only valueless as a food but actually harmful.

Magendie's Report in 1841 to the Paris Academy on the results of the investigations of the second Gelatin Commission was scarcely more favourable. Gelatin was considered to have no food value by itself and to reduce the value of other foodstuffs when fed in combination with them. A similar opinion was expressed by the Academy of Medicine in 1850 but less extreme views were held by some physiologists among whom were Boussingault [1846] and Frerichs [1845]. The latter ascribed to gelatin the same significance as that of the "Luxus" protein, *i.e.* the excess protein in the diet over the requirements of the body as represented by the protein decomposition during starvation. Though unable to replace the body protein gelatin could, he held, be utilised in the same way as the nitrogen-free foodstuffs ("Respirationsmitteln").

Somewhat similar views were held by Bischoff [1853] while Donders [1853] considered that gelatin might reduce the body's needs for protein since these are not restricted merely to the replacement of tissues.

Voit's own experiments and those carried out in conjunction with Bischoff on dogs form the first systematic study of the nitrogen balances on diets containing varying quantities of meat, gelatin and fat. As the result of a large number of experiments he concluded that gelatin always spares protein and in a greater degree than fat or carbohydrate, but that gelatin plus fat reduces the protein decomposition more than does gelatin alone. On the other hand, however much gelatin and fat are given, body protein will be lost.

The energy requirements of the animal were not sufficiently considered in these investigations and a large part of the so-called sparing action of gelatin can be ascribed to its ability to furnish energy and so to reduce the use of body protein for this purpose. I do not suggest that the whole effect is to be explained in this way, but to what extent gelatin can satisfy any portion of the dog's specific nitrogen requirements cannot be ascertained from Voit's results.

During the next thirty years experiments upon dogs by feeding with gelatin were also carried out by Oerum [1879], Pollitzer [1885], Munk [1894], Kirchmann [1900] and Krummacher [1901]. In Oerum's experiments the dog received a basal diet of starch, butter and meat extract equivalent to over 80 calories per kilo body weight. During successive periods of from four to eight days this diet was supplemented by meat or the equivalent amount of gelatin. Unfortunately only the urea nitrogen was determined (by Liebig's titration method) and the faeces were not analysed, so that a nitrogen balance sheet cannot be made out, but the results appear to indicate that gelatin can save about half the amount of nitrogen excreted by a dog when receiving a carbohydrate diet of sufficient calorie value.

Pollitzer also gave his dog abundant carbohydrate to which, during successive periods, were added equivalent quantities of meat, digestion products of meat (peptone, etc.) and gelatin. Positive nitrogen balances were obtained with all except gelatin.

Kirchmann determined the amount of body protein spared by different amounts of gelatin, no other food except water being given. Taking the nitrogen output during starvation as 100 he found a saving of 25 % when the gelatin given was sufficient to satisfy only 7.5 % of the energy requirements of the animal, while eight times this amount was required to save 35 %. He estimated that a maximum saving of 39 % might be expected if the amount of gelatin could be increased to meet these energy requirements in full.

Krummacher continued these experiments with still greater quantities of gelatin, and obtained a result closely agreeing with Kirchmann's calculated figures. It is clear however that unless we also know the minimum nitrogen output when the energy requirements are fully met by nitrogen-free food-stuffs, the above relationships offer no evidence as to the capacity of gelatin to satisfy any of the specific nitrogen requirements of the animal. This value was not determined by either Kirchmann or Krummacher.

Munk's experiments were on a different plan from those mentioned above.

He gave a dog a diet of rice, fat and meat equal to 58 calories per kilo and containing 0.6 g. nitrogen per kilo body weight, which was more than twice the starvation output. He was then able to replace five-sixths of this protein by the equivalent amount of gelatin and still keep the animal in nitrogen equilibrium.

Kauffmann [1905] carried out a series of experiments on a similar plan but with the precaution of reducing the nitrogen intake of the standard diet to a much smaller amount than that given by Munk. This standard diet consisted of milk, rice, caseinogen (plasmon) and fat and was given in amount equal to 0.32–0.39 g. N and 63–72 calories per kilo body weight. Not more than one-fifth of the nitrogen of this diet could be replaced by gelatin nitrogen without an increase in the nitrogen output occurring. Kauffmann also investigated the possibility of improving the value of gelatin by supplementing it with tyrosine, tryptophan and cystine and concluded from his experiments on dogs and on himself that with these additions gelatin becomes of equal value with caseinogen.

At a much earlier date Escher [1876] had fed dogs and pigs with gelatin supplemented by tyrosine and found that their body weight was maintained, but Lehmann [1885] was unable to obtain this result in experiments on rats.

Rona and Müller [1906] carried out a series of very careful experiments with dogs on the same plan as those of Kauffmann but were unable to confirm the latter's conclusions. Their standard diet gave 0.2 g. N and 91 calories per kilo body weight. With this the animal was in nitrogen equilibrium, but when a portion of the milk was replaced by gelatin plus tyrosine and tryptophan a negative balance was found.

The value of gelatin fed in conjunction with other proteins has also been investigated by Murlin [1907, 1] both by experiments on dogs and on himself. With dogs on a diet containing one-fourth more than the fasting requirement of nitrogen, half of this being in the form of cracker meal and half in the form of caseinogen, it was not possible to replace the caseinogen nitrogen by gelatin nitrogen without increased loss of body protein. With other diets however, in which the protein was in the form of meat, up to 58 % could be replaced without loss of body protein. The fuel value of all diets was greater than the energy requirements of the animal but Murlin attributes the high replacement value obtained in some diets largely to the greater proportion of calories supplied by carbohydrate in place of fat.

It is possible that this factor may have influenced the result, though according to Zeller [1914] the nitrogen requirements are not affected by the proportion of fat to carbohydrate in the diet so long as this does not become greater than about 4 : 1.

There seems however to be insufficient reason for assuming that the amount of meat given in some of these diets was the minimum required for nitrogen equilibrium, and unless this were so the fact that a part could be replaced by gelatin without affecting the balance would prove nothing.

On the other hand, on the cracker meal diets a negative balance was always obtained, which was recognised by Murlin as evidence of the lower availability of this form of protein. On these diets it was not possible to replace any part of the protein by gelatin without increasing the relative loss of body nitrogen.

The criticism that the protein in the diet after part of it had been replaced by gelatin may have still been in excess of the minimum required, applies even more forcibly to the experiment on himself, in which the basal diet contained 14.25 g. N, *i.e.* about 10 % more than his nitrogen output during starvation. When two-thirds of this had been replaced by gelatin nitrogen he was still receiving 5.33 g. N (0.076 g. per kilo) derived from eggs, cream, butter and cereals. During the two days on which this diet was taken a positive balance was obtained, but this cannot be accepted as convincing evidence of the value of gelatin nitrogen.

In a later paper Murlin [1907, 2] brought forward satisfactory proof that in a dog the reduction (about 30 %) of the fasting nitrogen output produced by small amounts of gelatin, was much greater than could possibly be accounted for by the dextrose which might be synthesised in the body from this gelatin¹.

From the investigations so far considered it may be taken as definitely established that:

1. Gelatin when given as the sole source of nitrogen is unable to maintain the animal body in nitrogen equilibrium.

2. With dogs gelatin is able to reduce the loss of body nitrogen considerably below that occurring during starvation, and this effect is not proportional to the amount of potential energy thus supplied and cannot therefore be simply explained on these grounds.

3. Some of the experiments indicate that when gelatin is mixed with other proteins, they may complement one another so that a proportion of the nitrogen of gelatin is utilisable.

A critical examination of the results of these experiments does not enable us to form any definite conclusions as to the capacity of gelatin alone to satisfy any part of the specific nitrogen needs of the body in man, although some of the results obtained with dogs indicate a limited capacity in this direction if the nitrogen output on an abundant nitrogen-free diet is taken as representing these specific requirements. There is however a difficulty in accepting this since the results obtained with dogs do not fall into line with those obtained with man and some other animals, and suggest that the nitrogen metabolism of the carnivora varies from that of the omnivora and herbivora in some details.

The fasting output of a man is equal to about 0.2 g. N per kilo body

¹ A brief account of other researches by Ganz, Gerlach (1891), who investigated the value of gelatin peptones, Gregor (1901), who used gelatin for feeding infants, and by Brat (1902) and Mancini (1905), who fed it to convalescents, will be found in Murlin's paper [1907, 1].

weight, that of a large dog is of the same order. On an abundant carbohydrate diet the nitrogen output of man can be reduced to one-quarter of this amount, *i.e.* 0.05 g. N per kilo whereas according to most observations under the same circumstances the nitrogen output of a dog is only reduced by 10 % to 20 %.

The difference in detail between the nitrogen metabolism of man and dog also emerges on comparison of the ratios of the total nitrogen to that excreted in the form of creatinine during starvation and on abundant nitrogen-free diets (see Table I). The constancy of the creatinine output and its probable relationship to the endogenous metabolism has been noted by Folin [1905], McCollum [1911], Zeller [1914] and others.

Table I

Observer	Animal	Weight Kg.	Diet	Total nitrogen in urine per kilo body weight	Creatinine nitrogen per kilo body weight	Total urine N Creatinine N
Cathcart [1907]	Man V.B.	62.0	Fasting, 4th day	0.221	0.0056	39
" "	" "	60.0	" 8th "	0.159	0.0053	30
Benedict and Osterberg [1914]	Dog 39	7.6	" 3rd "	0.360	0.0099	36
" "	" 33	12.7	" 3rd "	0.280	0.0112	25
Towles and Voegtlin [1912]	" 3	9.0	" 2nd "	0.294	0.0129	23
Murlin [1907, 2]	" C	13.0	" 4th "	0.257	0.0080	32
Folin [1905]	Man H.B.H.	85.7	Starch, cream, 1 g. N	0.0420	0.0070	6.0
Graham and Poulton [1912]	" G.G.	62.4	Starch, cream, .912 g. N	0.0445	0.0093	4.8
" "	" E.P.P.	72.4	Starch, cream, 1.23 g. N	0.0468	0.0107	4.4
af Klercker [1907]	" a.K.	88.0	Low N	0.0319	0.0079	4.0
Robison [1922]	" C.J.M.	60.5	Carbohydrate, fat, .3 g. N	0.0352	0.0072	4.9
" "	" R.R.	58.0	" "	0.0355	0.0084	4.2
McCollum [1911]	Pig	10.9	Carbohydrate	0.0495	0.0095	5.2
" "	" "	68.4	" "	0.0387	0.0069	5.6
Mendel and Rose [1911]	Rabbit	1.74	" "	0.126	0.0172	7.3
Murlin [1907, 2]	Dog C	11.3	" "	0.158	0.0104	15

There is a close parallelism between the figures for men and dogs during starvation and between those for men, pigs and rabbits on abundant nitrogen-free diets. The creatinine excretion for Murlin's dog C on such a diet is also in good agreement with the corresponding figures for men and pigs but the ratio $\frac{\text{Total urine N}}{\text{Creatinine N}}$ is about three times as high as the same ratio for other animals. It is of course not possible to state on such evidence alone that the real endogenous metabolism of this dog should be represented by a nitrogen output of one-third the observed amount, but it is clear that the nitrogen metabolism of dogs differs in some way from that of man, and that caution must be used in applying conclusions from experiments with these carnivora to other animals and man.

These criticisms however do not apply to the experiments of McCollum [1911] on pigs, for in these the constancy of the proportion of the endogenous

metabolism represented by creatinine nitrogen was recognised and was used as a criterion for judging when the minimum nitrogen excretion of the animals had been reached.

The pigs were fed on a basal nitrogen-free diet of ample fuel value consisting of starch, a salt mixture and water, until the nitrogen output had reached the minimum, whereupon an amount of the protein under examination equivalent to this minimum (urine nitrogen only) was added to the diet during a further period, after which the basal diet alone was fed until the output had again fallen to the minimum, the nitrogen excreted during this last period being also included in the calculation. In the experiment recorded by McCollum 2.62 g. of gelatin nitrogen was given daily during eight days, *i.e.* 20.96 g. in all. The total output during these eight and the following four days on which no nitrogen was given, amounted to 41.71 g. in the urine and 12.48 g. in the faeces, *i.e.* 54.19 g. in all, making a negative balance of 33.23 g.

Had the pig received no nitrogen at all its total output during these twelve days would have amounted to 31.44 g. in the urine and 12.48 g. in the faeces, making 43.92 g. in all, so that a saving of 10.69 g. nitrogen has been effected by 20.96 g. of gelatin nitrogen. This implies a utilisation of 50 % of the nitrogen given in this form, which was confirmed by five other similar experiments the details of which are not given. If the result is stated in terms of body protein saved, this amounts to 1.34 g. per day (if reckoned on eight days), *i.e.* 37 % of the minimum output in urine and faeces or 51 % of that in the urine only, which is taken by McCollum as representing the essential tissue metabolism of the animal.

Boruttau [1919] has recently attempted to determine the biological value of gelatin by two experiments on dogs, using the method and formulae adopted by Karl Thomas [1909] and has obtained the figures 49.1 % calculated by formula I and 67.3 % calculated by formula II. These values would agree much more closely had Boruttau not made an error in his use of formula I by taking the total food nitrogen as denominator in place of this amount less the nitrogen of the faeces, as intended by Thomas. In any case however such figures have no real significance in the case of gelatin since they will necessarily vary with the amount of the intake, and moreover the experiments were of too short duration to possess much value.

Apart then from the experiments of McCollum no very satisfactory evidence has been produced regarding the value of gelatin alone to satisfy any of the nitrogen requirements of the animal body. Most of the investigations have in fact been concerned with its value when fed in conjunction with other proteins and this introduces the possibility of complementary effect, about which very little is definitely known. That such effect is possible is shown by the experiments of Osborne and Mendel [1912] on rats. With gelatin as the sole protein the animals rapidly declined in weight but recovered when half of the gelatin was replaced by gliadin, a protein incapable of inducing more

than a very slight growth when fed as the sole protein constituent of the diet. Further, almost all the previous work, including that of McCollum, has been carried out on animals, and the results might not necessarily apply to man. The whole question is of very great theoretical and practical interest because of its bearing on protein metabolism in general and the nature of the body's requirements for particular compounds of nitrogen.

EXPERIMENTAL.

The investigation about to be described was an attempt to obtain more light on the problem by direct experiments on man.

The subject of the experiment was myself, age 37 years, medium build, weight 59 kilo, height 173.5 cm. My minimum nitrogen output had been determined by previous experiments which will be discussed in another paper.

In the second of these experiments, in which a diet containing about 0.3 g. N and equivalent to 2600–3000 calories (45–52 cals. per kilo) was taken for a period of seven days, the nitrogen output in the urine fell to a fairly constant level of 2.06 g., while the average amount of nitrogen excreted in the faeces was 1.13 g. per day.

In the present investigation the basal diet supplemented by different quantities of gelatin was taken for periods of ten days, the nitrogen intake being kept absolutely constant during each period.

Profiting by the experience of the previous experiments the basal diet was somewhat altered, the original attempt to introduce some variety and palatability being given up in favour of greater simplicity and uniformity of the food intake. The proportion of calories supplied by fat and the total nitrogen in the diet were both reduced. In the later experiments the process of simplification was carried to its furthest extent, the diet consisting of corn starch, lactose, sucrose and a salt mixture. Minimal quantities of lemon juice and cod liver oil were added to supply the antiscorbutic and fat soluble *A* accessory factors and agar-agar was taken to increase the bulk of the faeces and prevent constipation. The corn starch, lactose, salt mixture and agar for each day's ration were weighed out and mixed together before the experiment began. The mixture was taken in the form of a cream made with cold or warm (but not boiling) water and washed down with more water. The uncooked starch grains were very well absorbed, extremely few being found in the faeces. Usually a third of the day's ration was taken at 8 a.m., 1 p.m. and 7 p.m., but sometimes it was found necessary to increase the number of meals in order to consume the prescribed amount. The gelatin was dissolved in warm water and taken either by itself or mixed with some of the starch and lactose. The lemon juice, sweetened with cane sugar, was taken as a drink and a little weak tea with lemon was also permitted. The very small amount of nitrogen in the tea was assumed to be due to caffeine and to be excreted unchanged in the urine. It was therefore always subtracted from the total nitrogen intake and from the output.

The salt mixture had the following composition:

Calcium diacid phosphate $\text{CaH}_4(\text{PO}_4)_2, \text{H}_2\text{O}$	20	Na	2.5 %
Calcium lactate $(\text{C}_3\text{H}_5\text{O}_3)_2\text{Ca}, 5\text{H}_2\text{O}$	30	K	13.5
Potassium hydrogen phosphate K_2HPO_4	30	Ca	7.1
Sodium dihydrogen phosphate $\text{NaH}_2\text{PO}_4, \text{H}_2\text{O}$	15	P	13.6
Magnesium carbonate MgCO_3	3	Mg	.85
"Iron carbonate"	2	Fe	.7

It was intended that 10 g. of this mixture with the addition of 5 g. sodium chloride should be taken daily. The amounts of calcium and phosphorus would then correspond with those recommended by Sherman as 50 % above the minimum requirements of the body for these elements [Sherman, Wheeler, and Yates, 1918; Sherman, 1920]. It was found necessary however to reduce these quantities to 6 g. and 4 g. respectively on account of the diarrhoea caused by the diet. The ash of the above salt mixture is markedly alkaline, a point of importance in view of the observations by McCollum and Hoagland [1913] on the increased nitrogen output caused by diets having an acid ash.

The urine was collected from 8 a.m. to 8 a.m. and stored under toluene. The faeces were collected over the whole period and mixed with dilute sulphuric acid, those passed during the morning being considered as belonging to the previous day. Owing to the fluid consistency of the faeces the use of markers was found to be impracticable, but in view of the regular evacuation of the intestines and the length of the experiment, no serious errors can have been introduced in this way. Estimations of nitrogen in urine, faeces and in all components of the diet were carried out by the Kjeldahl method in duplicate. Creatinine was estimated by the method of Folin.

The percentages of nitrogen found in the constituents of the diet and their fuel values are given in Table II.

Table II.

	Nitrogen per 100 g.	Calories per 100 g.
Gelatin (Coignet's "Extra." Gold Label)	14.16	324
Corn starch (a)	0.039	360
" " (b)	0.027	360
Dextrin	0.065	360
Agar-agar	0.242	—
Lactose	0.013	370
Sucrose	—	395
Butter	0.080	775
Cod liver oil	Not determined	930
Lemon juice (per 100 cc.)	0.067	40
Vermouth "	0.005	140
Tea infusion* "	0.008	—

* The strength of the tea infusion was kept as nearly constant as possible but the total nitrogen intake from this source was checked by removing an aliquot portion of all tea drunk during an experiment and estimating the nitrogen in the whole quantity.

Concerning the purity of the gelatin used in the experiments.

The source from which commercial gelatin is obtained and the methods employed in its manufacture are not likely to produce a pure product. One would expect to find it contaminated with traces of other animal proteins or their decomposition products. Such included impurities being colloids could not be removed by washing, and might conceivably possess a high value for the replacement of body nitrogen.

Kirchmann drew attention to the fact that the best French gelatin gave a slight precipitate with Millon's reagent and with potassium ferrocyanide and acetic acid, and claimed to have succeeded in removing the impurities to which these reactions were due. The unoxidised sulphur was also reduced from 0.387 % to 0.263 %. He considered that the difference between his own results and those of previous workers was to be attributed largely to the presence of this protein in their gelatin. One of his methods consisted in soaking the gelatin first in water, then in 10 % sodium chloride solution, again in water and finally in alcohol. Murlin [1907, 1], using the same methods, was unable to detect any improvement in the purity of the product.

The gelatin used in the present investigation also gave a slight positive reaction with Millon's reagent and with potassium ferrocyanide and acetic acid, and an attempt was therefore made to purify it by soaking it for 24 hours in $N/20$ HCl followed by $N/20$ NaOH, then for some days in running water. No appreciable reduction in the intensity of the colour produced with Millon's reagent was observed after such treatment.

Folin and Denis [1912] have recorded finding a trace of tyrosine in gelatin, using Folin's colorimetric method, and Dakin [1920] has recently obtained a similar result using a gravimetric method. He estimates the amount of tyrosine at about 0.01 % and considers that it cannot be an integral part of the gelatin molecule.

I attempted to estimate the amount of tyrosine present by means of Folin's method, using relatively large quantities of gelatin. The tyrosine in a sample of dried ox muscle was also estimated by the same method. The results are shown in Table III. Millon's reaction is not well adapted for colorimetric measurement but under suitable conditions it was found possible to make approximate determinations by comparing the colour with that developed by different amounts of pure tyrosine, and the results agree reasonably well with those obtained by Folin's method.

Table III.

	Tyrosine estimated by Folin's method	Tyrosine estimated with Millon's reagent
Gelatin (Coignet's extra)	0.57 %	0.6 % to 0.7 %
„ after purification	0.45	—
„ Swiss	0.55	—
Glue	1.47	—
Ox muscle	5.8	—

The accuracy of Folin's method has been called in question by Abderhalden [1913, 1, 2] who has suggested that other amino-acids, tryptophan, hydroxytryptophan and hydroxyproline give the same colour reaction. Of these the first two are not present in gelatin but Dakin estimated the amount of hydroxyproline as 14.1 %. Through the kindness of Prof. Leathes, F.R.S., who supplied me with a specimen of this amino-acid, I was able to test its behaviour with Folin's reagent and found that a slight colour developed under the conditions laid down by Folin and Denis for the estimation of tyrosine, but that the intensity was about $\frac{1}{3.75}$ of that produced by the latter compound.

A slight colour, similar to that given by hydroxyproline, was also obtained from a specimen of phenylalanine.

In the face of the results given by gravimetric methods it would be rash to assert that the gelatin actually contained 0.57 % of tyrosine, but the colour produced with Folin's reagent does not appear to be due to any of the other amino-acids known to be present. It is also probable that the same compound, tyrosine or other amino-acid, is the cause of the colour produced with Millon's reagent.

If the percentage of tyrosine is correct and if it is present as a constituent of another protein similar to ox muscle, the proportion of the latter in the gelatin would be about 10 %. This calculation however is based on too many assumptions to be of more than speculative interest.

Up to the present neither cystine nor any other compound containing sulphur has been isolated from gelatin though the presence of such unidentified compounds has been noted by Dakin [1920].

The gelatin used in these experiments after purification in the manner described above, contained 0.24 % of total sulphur, calculated on the dry substance. Krummacher [1903] after purifying gelatin by Kirchmann's method found 0.28 % S (of which 0.02 % was in the form of SO_3 and SO_4). The original commercial product used by Krummacher contained 0.62 % total S, of which 0.4 % was present as SO_3 and SO_4 . Such an amount (0.24 %) of unoxidised sulphur would correspond with 0.9 % of cystine (or other compound containing a like proportion of S) and this can hardly be ascribed to impurities in the gelatin.

RESULTS OF THE EXPERIMENTS ON GELATIN DIETS.

It was proposed to carry out three diet experiments in which low, medium and high amounts of gelatin-nitrogen should be given in addition to the basal diet, in order to determine

- (1) whether the minimum nitrogen loss on abundant nitrogen-free diet can be still further reduced by gelatin, and if so,
- (2) what relation the amount of this reduction bears to the amount of gelatin ingested.

Two experiments were completed during the early part of 1921, but the

third had to be broken off through illness shortly after it was begun. It was repeated in September 1921 the conditions being somewhat modified on account of certain results that had in the meantime been obtained from other experiments carried out with Prof. C. J. Martin. These appeared to indicate that the amount of certain proteins required for nitrogen equilibrium could be greatly reduced if a carbohydrate diet very much in excess of the energy requirements was taken. In this last experiment, therefore, I increased the fuel value of the diet to the maximum that could be tolerated, so that the body weight was maintained and even increased during the first half of the period (see Fig. 1, Curve C). Synthesis of fat from the carbohydrate of the food was also indicated by the high respiratory quotient.

The diets for the three experiments are given together in Table IV. The diet taken during the experiment in which my minimum requirements were determined is also included for purposes of comparison.

Table IV.

Food	Nitrogen minimum 29/11/20—5/12/20 Total calories=2605 Calories per kilo=45 ¹ Calories supplied as Fat=31%.		Gelatin I 28/1/21—6/2/21 Total calories=2525 Calories per kilo=44 Calories supplied as Fat=6.4%.		Gelatin II 18/4/21—27/4/21 Total calories=2757 Calories per kilo=47 Calories supplied as Fat=6.5%.		Gelatin III 8/9/21—17/9/21 Total calories=3256 Calories per kilo=54 Calories supplied as Fat=0.86%.	
	Wt g.	N g.	Wt g.	N g.	Wt g.	N g.	Wt g.	N g.
Gelatin	—	—	66.67 ²	12.000	27.14 ²	4.885	41.89 ²	7.540
Corn starch	280	0.118	350	0.137	340	0.143	500	0.135
Dextrin	50	0.033	16	0.010	—	—	—	—
Butter and margarine	105	0.074	20	0.016	20	0.002	Cod oil 3	—
Honey	55	0.013	—	—	—	—	—	—
Sucrose	25	—	—	—	70	—	30	—
Lactose	65	0.008	180	0.023	250	0.032	300	0.039
Lemon juice	30 cc.	0.020	25	0.017	25	0.017	20	0.013
Vermouth	25 cc.	0.001	50	0.002	—	—	—	—
Tea	1200 cc.	0.072	350	0.028	250	0.020	600	0.043
Agar-agar	15	0.036	13	0.031	10	0.024	10	0.024
Salt mixture	5	—	10 ³	—	10 ³	—	10 ³	—
Total fluid	—	0.375	—	12.264	—	5.123	—	7.794
	—	—	2200—2500	—	2000	—	2200	—

¹ During the first five days of the experiment the fuel value of the diet was equal to about 52 cal. per kilo.

² Weight calculated as dry gelatin.

³ Includes 4 g. sodium chloride.

No purification was attempted for experiment I. For II and III the gelatin, after purification by the method described in the text, was dissolved in hot water. Weighed amounts of this solution were transferred to bottles and sterilised in the autoclave. The figures for the nitrogen intake are based on a number of analyses of samples from different bottles.

Gelatin I (28th Jan. to 6th Feb. 1921).

During the three days previous to the experiment a mixed diet containing about 12 g. N was taken.

The experimental diet is given in Table IV. It included:

N in the form of gelatin	...	12.00 g.
N in accessories ¹ (excluding tea)	...	0.23
Total N	...	12.23

¹ The nitrogen in the constituents of the basal diet.

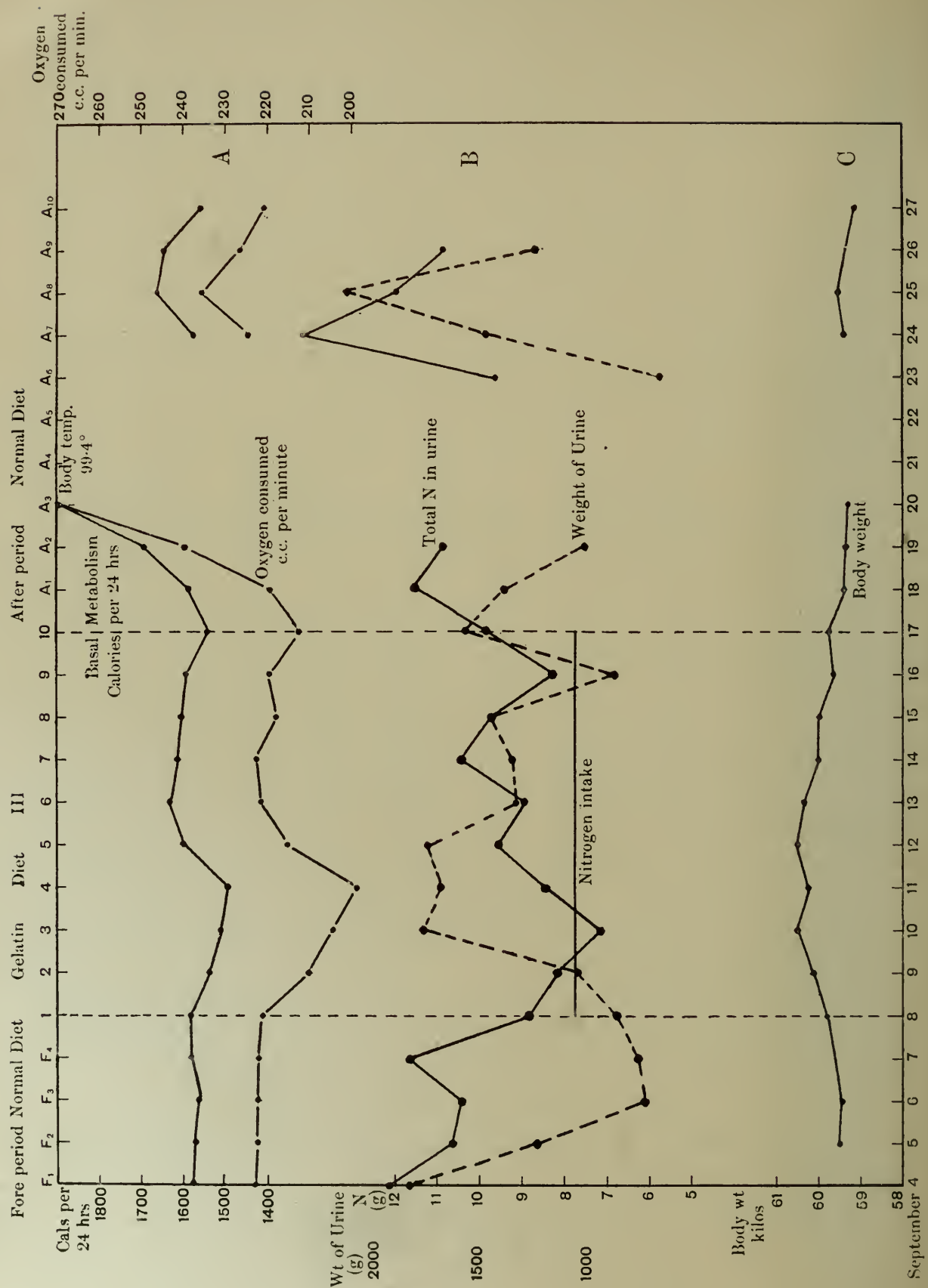


Fig. 1.

A few nitrogen-free biscuits made from starch, dextrin and agar were chewed at each meal to increase the flow of saliva. The rest of the starch etc. was taken in the raw state as already described.

The results of the experiments are shown in Table V. It will be seen that the nitrogen output in the urine remained almost stationary for three days, then rose and a series of oscillations set in.

Table V.

Date	Day of exp.	Body weight k.	Weight of urine g.	Sp. gr. of urine g.	Creatinine N g.	Total N in urine ¹ g.	N in faeces g.	Total N (U + F) g.	Balance
Jan. 28	1	58.75	1447	1.0175	0.44	12.37	(1.38) ²	(13.75)	(-1.50)
29	2	—	1790	1.014	0.53	12.42	1.38	13.80	-1.57
30	3	—	714	1.023	0.46	12.38	1.38	13.76	-1.53
31	4	—	1236	1.015	0.48	14.05	1.38	15.43	-3.20
Feb. 1	5	—	1550	1.013	0.52	12.84	1.38	14.22	-1.99
2	6	—	1496	1.015	0.53	14.42	1.38	15.80	-3.57
3	7	57.80	1200	1.016	0.51	13.13	1.38	14.51	-2.28
4	8	—	1300	1.015	0.53	14.20	1.38	15.58	-3.35
5	9	—	1601	1.014	0.50	12.27	1.38	13.65	-1.42
6	10	57.55	1914	1.014	0.54	14.44	1.38	15.82	-3.59
Average for whole period	0.50	13.25	1.38	14.63	-2.40
Average for the last six days	0.52	13.55	1.38	14.93	-2.70

¹ The "caffeine N" = 0.03 g. (from tea) has been subtracted from the total urinary nitrogen.

² The faeces were only collected for the last nine days.

Gelatin II (15th–27th April 1921).

During the two days previous to this experiment the diet consisted of eggs, milk, bread, potatoes, butter and apples, and contained 11.2 g. nitrogen. The experimental period was divided into two parts.

During the first three days the basal diet was supplemented by 250 g. of egg-white while during the last ten days this was replaced by gelatin. The two diets contained:

	Three days 15th–17th April	Ten days 18th–27th April
N in form of egg-white ...	5.0	—
„ „ gelatin ...	—	4.88
N in accessories (excluding tea) .	.21	.22
Total N ...	5.21	5.10
Fuel value ...	2580 cal = 44 cal per kilo	2757 cal = 47 cal per kilo (6.5 % supplied by fat)

The results are shown in Table VI.

A pronounced negative balance occurred on the egg-white diet and the nitrogen output during the first few days of the gelatin period is slightly above the average of the last six days.

Gelatin III (8th–17th Sept. 1921).

During the preceding four days a mixed diet containing about 12 g. N and equal to about 2400 calories was taken. On the 7th Sept. 100 g. lactose and 100 g. starch were consumed in addition to the above, making the total calories 3130.

Table VI.

Date	Day of exp.	Body weight k.	Weight of urine g.	Sp. gr. of urine g.	Creatinine N g.	Total N in urine ¹ g.	N in faeces g.	Total N (U + F) g.	Balance
<i>First period:</i>									
April 15	E 1	59.3	1351	1.017	—	7.30	—	—	—
16	E 2	—	1155	1.019	—	5.91	—	—	—
17	E 3	—	962	1.019	—	6.09	—	—	—
<i>Second period:</i>									
April 18	1	58.85	1517	1.013	0.52	7.42	1.28	8.70	- 3.60
19	2	—	1779	1.012	0.56	6.32	1.28	7.60	- 2.50
20	3	—	904	1.018	0.55	6.85	1.28	8.13	- 3.03
21	4	—	1125	1.015	0.53	6.73	1.28	8.01	- 2.91
22	5	—	833	1.019	0.55	5.68	1.28	6.96	- 1.86
23	6	—	1148	1.017	0.54	7.12	1.28	8.40	- 3.30
24	7	—	1523	1.013	0.54	6.18	1.28	7.46	- 2.36
25	8	58.35	817	1.021	0.53	6.12	1.28	7.40	- 2.30
26	9	—	1209	1.015	0.52	6.77	1.28	8.05	- 2.95
27	10	—	1020	1.017	0.53	6.56	1.28	7.84	- 2.74
28	—	58.15	—	—	—	—	—	—	—
Average of whole period			0.54	6.57	1.28	7.85	- 2.75
Average of last six days			0.53	6.41	1.28	7.69	- 2.59

¹ The "caffeine N" = .02 g. (from tea) has been subtracted from the total urinary nitrogen.

The experimental diet is given in Table IV. It included:

N in the form of gelatin	7.54 g.
N in accessories (excluding tea)21
Total N	7.75

From Sept. 14th the fuel value was increased by 100 calories taken in the form of starch biscuits and honey. The increased nitrogen is negligible.

The daily exercise consisted of a walk of from five to seven miles.

The results are shown in Table VII.

Here as in Gelatin I the nitrogen output during the first four days is below the average for the whole period.

Table VII.

Date	Day of exp.	Body weight k.	Weight of urine g.	Sp. gr. of urine g.	Creatin- ine N g.	Total N in urine ¹ g.	N in faeces g.	Total N (U + F) g.	Balance	
Sept. 8	1	59.8	850	1.0225	0.59	8.77	1.54	10.31	- 2.56	
9	2	60.1	1038	1.0175	0.59	8.12	1.54	9.66	- 1.91	
10	3	60.5	1764	1.011	0.60	7.11	1.54	8.65	- 0.90	
11	4	60.25	1680	1.013	0.58	8.43	1.54	9.97	- 2.22	
12	5	60.5	1735	1.013	0.60	9.49	1.54	11.03	- 3.28	
13	6	60.35	1320	1.0165	0.59	8.87	1.54	10.41	- 2.66	
14	7	60.0	1347	1.0155	0.57	10.37	1.54	11.91	- 4.16	
15	8	59.93	1439	1.015	0.59	9.68	1.54	11.22	- 3.47	
16	9	59.65	859	1.0215	0.59	8.20	1.54	9.74	- 1.99	
17	10	59.78	1555	1.0145	0.60	9.84	1.54	11.38	- 3.63	
Average for the whole period				0.59	8.89	1.54	10.43	- 2.68
Average for the last six days				0.59	9.41	1.54	10.95	- 3.20

¹ The "caffeine nitrogen" = .04 g. (from tea) has been subtracted from the total urinary nitrogen.

BASAL METABOLISM.

My basal metabolism was determined each day while on the experimental diet and during the periods immediately before and after.

The method adopted was that of the Douglas bag, the expired air being analysed in Haldane's gas analysis apparatus. From the 4th to the 7th of September the estimation was made at 9 a.m., fasting, after a walk of one mile followed by a resting period of at least 30 minutes. All the remaining determinations were made between 7.30 a.m. and 8 a.m. on waking. The results are shown in Table VIII.

A slight decrease in the basal metabolism occurred at the commencement of the experimental diet but the normal level was regained by about the fourth day. This decrease coincides with the lower nitrogen output, as may be seen in Fig. 1 A and B, but it is not possible to say whether the two are in any way connected. No corresponding decrease occurred in the creatinire output which was constant throughout the period.

In calculating the basal metabolism in calories per 24 hours the protein oxidation has been ignored but the error thus introduced is less than + 1 %. For those days in which the R.Q. is greater than 1 a correction has been made for the nett heat production due to synthesis of fat from carbohydrate by adding 0.3 of the calories equivalent to the excess of the CO₂ output over the oxygen consumed. I am aware that this is an arbitrary estimate, but the possible error involved is only slight.

Table VIII.

Date	Day of exp.	Diet during previous 24 hours (calories per kilo)	Oxygen consumed per minute (cc)	R.Q.	Calories per 24 hours
Sept. 4	F 1	Normal (40)	223.0	0.831	1575
5	F 2	" "	222.5	0.830	1571
6	F 3	" "	222.4	0.803	1561
7	F 4	" "	222.3	0.866	1580
8	G 1	Normal + 200 g. starch (54) lactose mixture	221.2	0.904	1582
9	G 2	Carbohydrate + gelatin (54)	210.1	1.012	1537
10	G 3	" "	204.8	1.043	1510
11	G 4	" "	199.0	1.093	1492
12	G 5	" "	215.3	1.065	1600
13	G 6	" "	221.6	1.051	1633
14	G 7	" "	222.8	0.967	1614
15	G 8	" "	218.0	1.040	1604
16	G 9	" "	219.8	0.967	1592
17	G 10	" "	212.9	0.968	1542
18	A 1	" "	218.7	0.979	1587
19	A 2	Normal diet	239.8	0.823	1691
20	A 3	" "	269.3 ¹	0.805	1893
24	A 7	" "	224.3	0.801	1574
25	A 8	" "	235.5	0.809	1660
26	A 9	" "	226.3	0.823	1596
27	A 10	" "	220.9	0.830	1559

¹ The body temperature was 99.4° when this determination was made. Several attacks of vomiting had occurred during the previous night. The condition became worse and necessitated some days' rest.

The normal *basal metabolism* calculated from Harris and Benedict's formula for a man of age 37, weight 59.8 kilo, height 173.5 cm. would be $66.4730 + 13.7516 \times 59.8 + 5.0033 \times 173.5 - 6.755 \times 37 = 1506$ calories.

GENERAL CONSIDERATIONS.

My usual mode of life was followed throughout these experiments, eight or nine hours of each day being occupied with laboratory work. The only form of exercise was a walk of from two to seven miles. No great difficulty was found in consuming the diet although a feeling of nausea was frequently experienced. This was greatly intensified by the excessive quantity of food in the third experiment; the tongue became furred and more or less headache was common. Slight diarrhoea occurred in all three periods, faeces of fluid consistency being passed two or three times a day. The diet was however very well assimilated scarcely any starch being found in the faeces. Traces of reducing sugar were regularly present in the urine during the third experiment but only occasionally during the first two.

It was intended that a period on nitrogen-free diet should follow immediately on the third gelatin diet in order to determine my minimum requirements once more. This was not possible, and indeed much difficulty was experienced in completing the ten days proposed for the above experiment.

In the two experiments (*G I*, *G III*) which followed a normal mixed diet the average nitrogen output during the first four days was lower than that for the remainder of the period while the reverse of this was observed when the previous diet had been insufficient to satisfy the protein requirements (*G II*) and a considerable negative balance had occurred. These differences are probably due to the influence of the protein of the preceding diet, a diminishing store of which, perhaps in the form of amino-acids, remains in the body for some days. The first four days have therefore been excluded in considering the results. Another disturbing factor is the variation in the urine nitrogen from day to day. This frequently amounted to more than 20 % of the average output and showed no relationship whatever to the volume of the urine. Consequently it cannot be explained by diuresis.

SUMMARY AND DISCUSSION OF RESULTS.

The results obtained in the three experiments are summarised in Table IX, the average figures being given for the last six days of each ten day period. The average amounts of the nitrogen intake and output on the last three days of the earlier experiment on "nitrogen-free" diet are also included.

Table IX

Date	Body weight (kilos)	Fuel value of diet, cals per kilo	Nitrogen intake		Nitrogen output				Balance
			Gelatin g.	Accessories g.	Urine g.	Faeces g.	Total g.	Creatinine g.	
3. xii. 20-5. xii. 20	58.0	52-44	—	.30	2.06	1.13	3.19	0.49	-2.89
1. ii. 21-6. ii. 21	57.8	44	12.00	.23	13.55	1.38	14.93	0.52	-2.70
21. iv. 21-27. iv. 21	58.4	47	4.88	.22	6.41	1.28	7.69	0.53	-2.59
11. ix. 21-17. ix. 21	60.2	54	7.54	.21	9.41	1.54	10.95	0.59	-3.20

In attempting to calculate the amount of body protein spared by the gelatin from these results we are met by two difficulties, namely what is to be done with that part of the intake due to the nitrogen of the accessories, and with that part of the nitrogen output due to the faeces.

Nitrogen of the accessories. The nitrogen from the tea does not appear in the table, having been subtracted both from intake and output. The assumption that this is all "caffeine N" is not strictly correct but as the total amount is very small, usually under 0.05 g. any error involved in this mode of treatment must be negligible. The greater part of the accessory nitrogen comes from the corn starch. In McCollum's account of his experiments on pigs, no mention is made of any nitrogen arising from this source although large quantities (1700 g.?) of corn starch were given. If this starch contained as much nitrogen as the samples used by me the nitrogen intake from this source may easily have been 0.5 g. or more. We do not know in what form this nitrogen is present nor its value in the human body and cannot therefore estimate its effect on the nitrogen output. If the amount and nature of such accessories are the same during the determination of the nitrogen minimum and experiments with gelatin, the following argument might be applied. If the value of this nitrogen in the accessories is zero then the real nitrogen requirements will be less than the observed output by the full amount of such nitrogen intake since the latter must be excreted in addition to the nitrogen resulting from the protein metabolism of the body. If the value of this nitrogen for the replacement of body nitrogen is 100 % then it will spare an equal amount of the latter and the observed output on the so-called "nitrogen-free" diet will represent the actual minimum requirements. But in this case an equal amount of gelatin will also be spared when gelatin is taken, and the apparent sparing effect of the gelatin will thus be increased by the same amount.

In either case the real saving of body nitrogen due to the gelatin will be less than the apparent saving, *i.e.* the difference between the negative balance on the gelatin and the minimum nitrogen output on the "nitrogen-free" diet, by an amount equal to the nitrogen of the accessories. This will also hold for all values of the latter between 0 and 100 %. Unfortunately the proviso that the accessories should be the same on both diets does not strictly hold in the above experiments but if the butter nitrogen be subtracted from the total intake on the "nitrogen-free" diet the remainder is practically the same as the accessory nitrogen on the gelatin diets. The butter nitrogen would probably have a high value and I have therefore assumed that it does not appreciably increase the nitrogen output. The rest of the accessory nitrogen (0.22 g. average) has been deducted in calculating the amount of body nitrogen saved by the gelatin. This is not strictly accurate but is probably the best that can be done with the figures. The above argument however ignores the possibility of complementary action of the accessories and the gelatin. It has been already pointed out that such action occurs when gelatin is fed with certain cereal proteins. McCollum, Simmonds and Pitz [1917] have shown that a mixture of oat protein and gelatin has a higher value than either alone or than oat protein plus caseinogen. It is impossible to say whether the results in my experiments were affected by such complementary action but if this did occur the real value of the gelatin alone is still less than the calculations appear to show.

Faecal nitrogen. The problem of how to treat the nitrogen excreted in the faeces is even more difficult. In McCollum's experiments the urinary nitrogen is alone considered, that in the faeces being estimated merely as a check on the complete absorption of the food protein. He considers that the nitrogen of the urine represents the essential tissue metabolism, while that of the faeces represents losses which may be termed accidental in character.

It has been shown by Rubner [1919] that the increased amount of nitrogen in the faeces of men, when fed on various diets, above that found on a carbohydrate diet cannot be taken as entirely due to undigested food protein, but that a considerable proportion of the increase comes from the body. In my experiments somewhat large variations were observed in the nitrogen of the faeces and these may have been due to the slight diarrhoea which occurred. Probably most of the nitrogen came from the body but the possibility that a small amount of gelatin escaped absorption must not be overlooked. We know very little about the relationship between loss of body nitrogen through the intestines and that excreted in the urine, but there seems to be no evidence that an increase in the former is accompanied by a decrease in the latter. The reverse of this is perhaps more probable.

This question remains at present the limiting factor for the accuracy of such experiments. I have attempted to define the limits between which the true conclusion from my results is to be found, by calculating the amount of body nitrogen saved by the gelatin in two ways. In the first (A) I have assumed that my minimum nitrogen requirements are represented by the sum of the nitrogen in the urine and that in the faeces on the nitrogen-free diet, and that the difference between the latter amount and the corresponding excretion on the gelatin diets represents unabsorbed gelatin nitrogen.

Table X.

Experiment	N Intake			A			B		
	Gelatin g.	Accessories g.	N Balance g.	Body Nitrogen saved			Body Nitrogen saved		
				N minimum g.	g.	% of minimum	N minimum g.	g.	% of minimum
R.R. II	4.88	0.22	-2.59	3.19	0.38	11.9	3.34	0.53	15.9
R.R. III	7.54	0.21	-3.20	3.19	0	0	3.60	0.19	5.3
R.R. I	12.00	0.23	-2.70	3.19	0.26	8.1	3.44	0.51	14.7
McCollum's pig average of last six day	2.62	?	-2.35	3.68	1.33	36.1	3.66	1.31	36.3

In the second (B) I have assumed that the gelatin is completely absorbed and that the minimum requirements for such periods are represented by the output in the urine on "nitrogen-free" diet plus the nitrogen in the faeces on the gelatin diet under consideration. The truth probably lies somewhere between these two extremes. The results of these calculations are given in Table X. McCollum's figures are also included for comparison. The accessory nitrogen has been in each case deducted from the apparent amount of body nitrogen saved.

The maximum saving in terms of the nitrogen minimum is thus 11.9 % if the first method of calculation is employed and 15.9 % if the second is used.

The fact that this was obtained with the lowest amount of gelatin would appear to prove that the effect is not due to any impurity, in which case there should be a proportionality between the amount saved and the gelatin intake. In this connection however the possibility that the increased protein in the diet entails an increased loss of body nitrogen must not be overlooked, although the creatinine excretion does not lend any support to such an hypothesis.

The results of the first and second experiments agree well between themselves, but in the third a much lower value was apparently indicated. It will be noticed however that the creatinine excretion in the experiment was higher than the normal and the body weight was also higher. This nitrogen minimum may therefore have been higher than the amount shown (perhaps owing to the excessive amount of food taken) in which case the calculated result would be too low.

All these values are much lower than those found by McCollum and this discrepancy cannot be explained except by assuming a difference in the metabolism of man and pig. If the values are calculated in terms of the urinary output alone they are all proportionately increased—the value for my experiment *G* II then becoming 25.7 % of the minimum, but the difference between the man and the pig still persists.

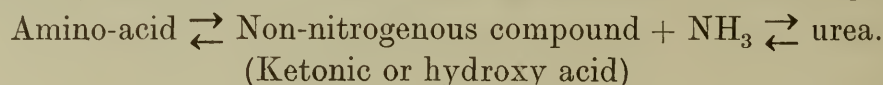
The creatinine output has been shown to bear some close relationship with the minimum nitrogen output. I therefore attempted to compare my results with McCollum's in terms of the amounts of creatinine excreted in the several experiments. I ignored the faeces and calculated the negative balance by subtracting the nitrogen intake from the output in the urine. The ratio of this balance to the average amount of creatinine nitrogen excreted during the same period is shown in the last column of Table XI. The last six days of each period have been alone considered.

Table XI.

Experiment	Nitrogen intake	Nitrogen in urine	Nitrogen balance	Creatinine N	Nitrogen balance Creatinine nitrogen
	g.	g.	g.		
R.R. II	5.10	6.41	-1.31	0.53	2.47
R.R. III	7.75	9.41	-1.66	0.59	2.81
R.R. I	12.23	13.55	-1.32	0.52	2.54
McCollum's pig	2.62	3.89	-1.27	0.48	2.65

There is obviously no discrepancy between our results when they are considered in this way, though what this agreement implies is not easy to state. It has been shown in many papers by Grafe (1912-1914), Abderhalden [1915], Underhill and Goldschmidt [1913] and others, that many nitrogen compounds other than amino-acids, namely organic ammonium salts, urea etc., have the capacity to spare a certain proportion of the loss of body nitrogen occurring on a carbohydrate diet. The results obtained by these workers do not agree in all points but the amount of nitrogen thus spared appears to be of the same order as that spared with gelatin in my experiments. It may well be that the action of the gelatin is of the same nature as that of these simpler

compounds and consists essentially in the reduction of the waste of amino-acids derived from body protein through deamination and subsequent oxidation in the body. The amino-acids of the gelatin and the ammonia and urea produced from them can play a part in the reversible reactions that are constantly proceeding in the body and thus influence the resulting equilibrium.



If this is true the loss of body nitrogen when both carbohydrate and gelatin are fed may represent a "N-minimum" that corresponds more closely with the specific nitrogen requirements of the body than does the output on carbohydrate diet alone. This may perhaps be the explanation of the close agreement between the ratios of such loss to the creatinine nitrogen shown by my experiments and those of McCollum.

In conclusion I would express my very sincere thanks to Prof. C. J. Martin for his constant encouragement and advice throughout this investigation.

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XVII. DISTRIBUTION OF THE NITROGENOUS CONSTITUENTS OF THE URINE ON LOW NITROGEN DIETS.

By ROBERT ROBISON.

From the Lister Institute.

(Received January 17th, 1922.)

DURING the course of experiments carried out in conjunction with Prof. C. J. Martin on ourselves, with the object of determining our minimum nitrogen requirements and the biological value of certain proteins, the opportunity was taken of investigating the distribution of the nitrogenous constituents in the urine when the total nitrogen output had reached a very low level.

In the particular experiments referred to in this paper, a diet consisting of carbohydrate and fat (corn starch, sucrose, lactose, honey, butter) together with a little lemon juice, inorganic salts and agar-agar was taken during seven days. The fuel value of this diet was equal to 44–52 calories per kilo body weight and the nitrogen content was about 0.3 g. A little tea and coffee was also taken but the nitrogen in these beverages was assumed to be "caffeine nitrogen" and to be excreted as such in the urine during the same 24 hours. It was therefore subtracted from the total nitrogen before calculating the percentage amounts of the other constituents. The above assumption is of course not strictly accurate, but in the one experiment (R.R.) the total amount of such nitrogen is very small, less than 0.1 g., so that any error thus introduced may be considered negligible.

The period on this low nitrogen diet was immediately followed by another of five days during which the same basal diet with the addition of a little milk was taken, the nitrogen intake being thus raised to about 3 g.

The total nitrogen in the urine and in all constituents of the diet was estimated by Kjeldahl's method, urea by van Slyke's urease method, ammonia by Folin's aeration method, amino-acids by formal titration after removal of the ammonia, creatinine by the method of Folin, and uric acid by that of Hopkins as modified by Folin and Schaffer.

The results are set out in Table I. In Table II the distribution of nitrogen on the days for which the total nitrogen output reached its lowest values has been compared with corresponding figures obtained by other investigators.

It will be seen that these observations are in complete agreement with Folin's [1905, 2] generalisations respecting the variation in the distribution of the urinary nitrogen. The creatinine nitrogen is practically constant and is

Table I.

Subject: C.J.M. Weight Nov. 28th—61.9 kilos; Dec. 5th—60.5 kilos

Subject: C.J.M. Weight Nov. 28th—61.9 kilos; Dec. 5th—60.5 kilos																	
Date 1920 Dec.	Day of exp.	Intake N g.	Volume of urine cc. Sp. gr. of urine	Total N less caffeine		In percentage of total N (less caffeine N)											
				Total N g.	Urea N g.	Ammonia N g.	Amino- acid N g.	Creatin- ine N g.	Uric acid N g.	Undeter- mined N g.	Urea	Ammonia	Urea + Ammonia	Amino- acid	Creatin- ine	Uric acid	Undeter- mined
4	6	.35 .291	1421 1.014	2.40 2.69	1.27 2.40	.29 1.08	.05 2.13	.45 2.42	.07 2.57	.27 3.01	52.9 3.12	12.1 3.46	65.0 3.46	2.1 3.46	18.7 3.46	2.9 3.46	11.3 3.46
5	7	.33 .261	908 1.020	2.13 2.39	1.08 2.13	.21 1.08	.05 2.13	.43 2.42	.07 2.57	.29 3.01	50.7 3.12	9.9 3.46	60.6 3.46	2.3 3.46	20.2 3.46	3.3 3.46	13.6 3.46
6 ²	8	.37 .251	1708 1.011	2.67 2.74	1.24 2.57	.30 1.39	.11 2.23	.47 2.42	.02 2.06	.28 2.29	51.2 3.12	12.4 3.46	63.6 3.46	4.5 3.1	19.4 16.3	.8 2.3	11.5 15.2
7	9	.36 .171	1207 1.0165	2.57 3.30	1.39 3.01	.23 1.91	.08 2.06	.42 2.45	.06 2.06	.39 2.29	54.1 3.12	9.0 3.46	63.1 3.46	3.1 2.0	16.3 15.0	2.3 2.0	15.2 9.6
8	10	.42 .291	1060 1.020	3.01 3.30	1.91 3.01	.24 1.91	.06 2.06	.45 2.45	.06 2.06	.29 2.29	63.4 3.12	8.0 3.46	71.4 3.46	2.0 1.9	15.0 14.1	2.0 2.6	9.6 9.6
9	11	.40 .341	1128 1.0185	3.12 3.46	1.96 3.12	.28 1.96	.06 2.06	.44 2.44	.08 2.06	.30 2.29	62.8 3.12	9.0 3.46	71.8 3.46	1.9 1.9	14.1 14.1	2.6 2.6	9.6 9.6

Subject: R.R. Weight Nov. 28th—58.6 kilos; Dec. 5th—57.7 kilos.

3	5	.32 } .091 }	1886 1.007 }	2.08 } 2.08 }	1.99 2.08	.74 2.08	.04 2.08	.50 2.08	.07 2.08	.29 2.08	37.2 2.08	17.6 2.08	54.8 2.08	2.1 2.08	25.1 2.08	3.5 2.08	14.5 2.08
4	6	.30 } .071 }	1703 1.010 }	2.24 } 2.24 }	2.17 2.24	.98 2.24	.05 2.24	.50 2.24	.10 2.24	.27 2.24	45.2 2.24	12.4 2.24	57.6 2.24	2.3 2.24	23.0 2.24	4.6 2.24	12.4 2.24
5	7	.27 } .061 }	1547 1.009 }	2.07 } 2.07 }	2.01 2.07	.91 2.07	.04 2.07	.47 2.07	.07 2.07	.27 2.07	45.3 2.07	12.4 2.07	57.7 2.07	2.0 2.07	23.3 2.07	3.5 2.07	13.4 2.07
6 ²	8	.298 } .101 }	1783 1.010 }	2.59 } 2.59 }	2.49 2.59	1.26 2.59	.04 2.59	.50 2.59	.07 2.59	.31 2.59	50.6 2.59	12.5 2.59	63.1 2.59	1.6 2.59	20.1 2.59	2.8 2.59	12.4 2.59
7	9	.294 } .091 }	1991 1.011 }	3.04 } 3.04 }	2.95 3.04	1.71 3.04	.04 3.04	.50 3.04	.03 3.04	.38 3.04	57.9 3.04	9.8 3.04	67.7 3.04	1.4 3.04	17.0 3.04	1.0 3.04	12.9 3.04
8	10	.309 } .161 }	1403 1.012 }	3.38 } 3.38 }	3.22 3.38	2.06 3.38	.04 3.38	.45 3.38	.05 3.38	.36 3.38	64.0 3.38	8.1 3.38	72.1 3.38	1.2 3.38	14.0 3.38	1.5 3.38	11.2 3.38
9	11	.291 } .151 }	1134 1.014 }	3.01 } 3.01 }	2.86 3.01	1.69 3.01	.04 3.01	.45 3.01	.09 3.01	.30 3.01	59.1 3.01	10.1 3.01	69.2 3.01	1.4 3.01	15.8 3.01	3.1 3.01	10.5 3.01
10	12	.297 } .181 }	1260 1.015 }	3.01 } 3.01 }	2.83 3.01	1.89 3.01	.03 3.01	.46 3.01	.02 3.01	.17 3.01	66.8 3.01	9.2 3.01	76.0 3.01	1.1 3.01	16.2 3.01	.7 3.01	6.0 3.01

¹ N from tea and coffee—taken as "caffeine N."
² Milk diet began on Dec. 6.

Table II.

Table II.

Observer	Subject	Body weight	Day of exp.	Intake N g.	Total N g.	Urea N g.	Percentages of total N				Undeter- mined N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.	Amino-acid				Urea N g.	Uric acid N g.	Creatin- ine N g.
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¹ N from tea and coffee—taken as "caffeine N."
² Total urine N less "caffeine N."

equal to 7.3 mg. (C.J.M.) and 8.2 mg. (R.R.) per kilo body weight. These amounts like those of the other constituents are very similar to those found by Folin and others. The values for the total nitrogen are amongst the lowest on record and correspond with still further reductions in the percentage of urea nitrogen, this falling in one instance to 37 % of the total. The last figure was however accompanied by a somewhat high percentage of ammonia, but the sum of the urea and ammonia nitrogen was only 54.6 % of the whole amount. It would have been interesting to discover whether this sum (urea + ammonia) could have been further reduced by the ingestion of alkalies or whether any decrease in the ammonia nitrogen would have been accompanied by an increase in the urea.

The question as to whether any part of the urea nitrogen represents what Folin has termed the endogenous metabolism remains open.

I wish to express my indebtedness to Prof. C. J. Martin for his interest and help in this work.

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XVIII. THE ESTIMATION OF TOTAL SULPHUR IN URINE.

By ROBERT ROBISON.

From the Lister Institute.

(Received January 27th, 1922.)

THE methods for the estimation of total sulphur in urine are less satisfactory than those available for determining many of the other urinary constituents. A high degree of accuracy is specially important when we are concerned with the amount of unoxidised or "neutral" sulphur in the urine. This can only be determined by subtracting the amount of sulphates from that of the total sulphur and any error in the latter value falls on the relatively small difference.

Benedict's [1909] method in which the oxidation is effected with a mixture of copper nitrate and potassium chlorate has been criticised on the ground that considerable losses frequently occur through spattering during the initial stages of the reaction. Denis [1910] has stated that out of forty analyses mechanical loss resulted in every case. She has proposed to substitute a solution of copper nitrate, ammonium nitrate and sodium chloride in place of Benedict's oxidising reagent, thereby reducing the vigour of the reaction.

In my hands Denis' modification has not proved so reliable as the original method. Decrepitation is certainly less troublesome but the results are frequently too high, the errors amounting at times to more than 10 mg. The following series taken from nearly a hundred analyses are fairly representative:

25 cc. urine (1) gave 0.0711, 0.0719, 0.0783, 0.0826 g. BaSO_4 ,
,, (2) ,, 0.0864, 0.0901, 0.0865, 0.0899, 0.0877 g. BaSO_4 .

The residue, even after prolonged ignition always contained appreciable amounts of nitrate, this being probably due to sodium nitrate formed from the sodium chloride and copper nitrate since the latter is readily decomposed at temperatures lower than those used. Whether the presence of this nitrate would alone account for the high results I am not able to say. Considerable errors in this direction were observed by Kolthoff and Vogelensang [1919] using higher concentrations of both sulphate and nitrate.

Consistent results were usually obtained by Benedict's original method, using an electric hot plate as suggested by Givens [1917] although in spite of this precaution vigorous spattering sometimes occurred. Only slight traces of nitrate were found in the residues after ignition and it is probable that these arose from the small amounts of alkali salts in the reagent and in the urine itself. The necessity of igniting the residue at a red heat was however

a frequent cause of spoiled analyses through the cracking of the porcelain basins. Probably those used by Benedict were of better quality than are at present obtainable.

Errors due to the use of coal gas for igniting the residue.

Neither Benedict nor Denis appears to have recognised the possibility of errors from the presence of sulphur in the coal gas used for the ignition of the residue. Folin [1903] advised spirit burners for his original method and according to Liebesny [1920] errors of 8–10 % in the amount of neutral sulphur may arise from the use of gas. My own experience confirms this. In determining the blank of the oxidising solution referred to below, 2.5 cc. was found to yield 0.7 mg. BaSO_4 , using a spirit burner, and 2.4 mg. using an argand burner with coal gas. If this error were constant in amount it would be cancelled in subtracting the blank but such was not found to be the case. Probably the absorption of oxides of sulphur depends on the surface area of the residue as well as on the total quantity present in the gas.

The use of a spirit burner is therefore essential if accurate results are to be obtained.

Since copper nitrate, which forms the basis of Benedict's reagent, is decomposed at comparatively low temperatures it seemed possible that the necessity of igniting the residue at a red heat might be avoided if neither chlorate nor alkali salts were introduced. Other substances were therefore tried as a diluent for the copper nitrate and of these copper chloride was found to be the most satisfactory. In the presence of a suitable proportion of this salt spattering never occurs, while the nitrate can be completely decomposed by heating the residue over a small spirit stove of the simplest kind. The temperature of a good argand burner is sufficiently high but gas cannot be used.

The oxidising reagent finally adopted has the following composition:

Copper nitrate (cryst.)	40 g.
Copper chloride (cryst.)	15 g.
Water	to 100 cc.

2.5 cc. of this solution are added to 10 cc. of the urine in a 4-inch porcelain basin and evaporated to dryness on a water-bath or electric hot plate. The oxidation can be started on the hot plate, or over a very small spirit flame. It proceeds rapidly but smoothly, leaving a coherent residue which frequently swells up. The dish is then heated over a broad spirit flame for 20 minutes. A spirit stove of the common kind is suitable but a sound tin, half filled with methylated spirit answers very well. A better flame is obtained if a number of holes are punched about half-way up the tin. The residue is dissolved in 10 cc. of 2N HCl and diluted with 300 cc. distilled water. The sulphate is precipitated in the boiling solution with 10 cc. of a 5 % solution of barium chloride, dropped in very slowly by means of a dropping tube. The precipitate is allowed to stand overnight before being filtered.

In the following analyses the precipitates were weighed in platinum crucibles.

For 25 cc. urine, 6.25 cc. of the oxidising solution, 20 cc. 2*N* HCl, 400 cc. H₂O and 15 cc.–20 cc. of 5 % barium chloride solution were used. A blank determination must be carried out with the reagents.

Two series, each of eight estimations, of the total sulphur in a normal urine yielded the following results.

BaSO ₄ from 10 cc. urine		BaSO ₄ from 25 cc. urine	
1.	0.0451	1.	0.1140
2.	0.0454	2.	0.1143
3.	0.0460	3.	0.1135
4.	0.0454	4.	0.1141
5.	0.0456	5.	0.1138
6.	0.0457	6.	0.1133
7.	0.0461	7.	0.1141
8.	0.0454	8.	0.1148
Average 0.0456 g.		Average 0.1140 g.	
		equivalent to 0.0456 g. for 10 cc.	

It is doubtful whether any gain in accuracy is achieved by the use of 25 cc. instead of 10 cc. of urine.

In all the above estimations the filtrates were examined for nitrates by the very sensitive diphenylamine test, slight traces being found in one or two cases only.

The accuracy of the method was checked by estimating the total sulphur in two solutions of cystine containing also 2 % of urea and a definite volume of *N*/10 H₂SO₄ neutralised by an equal amount of *N*/10 NaOH.

The sample of cystine, for which I am indebted to the kindness of Dr J. C. Drummond, was analysed by the Carius method:

0.1878 g. gave 0.3679 BaSO₄; calculated 0.3681 g. BaSO₄.

BaSO ₄ from 10 cc. solution A		BaSO ₄ from 10 cc. solution B	
	0.0671		0.0662
	0.0670		0.0659
	0.0675		0.0661
	0.0675		0.0659
	—		0.0659
	—		0.0663
0.0673 g.		0.0661 g.	
Calculated 0.0678 of which		Calculated 0.0668 of which	
0.0212 is from cystine.		0.0202 is from cystine.	

This method has been employed for some time by myself and others in connection with metabolism experiments in this laboratory and has proved satisfactory.

I wish to thank Miss M. Tazelaar and Miss M. H. Carr for much valuable help in carrying out the numerous analyses, only a very small proportion of which have been recorded above.

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II

NOTES ON COPROZOIC FLAGELLATES

BY

H. M. WOODCOCK, D. Sc.

Fellow of University College.

1. On the presence of an accessory flagellum in the genus *Helkesimastix*,
Woodcock and Lapage.

In 1915, in collaboration with Dr. Lapage, I described¹ the life-cycle of a new type of coprozoic Flagellate, *Helkesimastix faecicola*, n. g., n. sp. This remarkable form is characterized by the possession of a long, trailing flagellum, in contact with the body of the Flagellate for part of its length. This disposition resembles that of the posterior flagellum of *Cercomonas*, and differs from that of the trailing flagellum of *Bodo* (syn. *Prowazekia*) and of *Heteromita*. Unlike these forms, however, *Helkesimastix* possesses no anterior, well-developed, vibratile flagellum, which, by its vigorous movement, causes the progression of the creature.

We looked carefully and often for another flagellum, because, as stated in our paper, it was very difficult to explain satisfactorily one variety of movement shown by this flagellate. We were unable, however, to determine the existence of any additional flagellum and in our account, therefore, we considered that there was only a single flagellum, namely, the long, trailing, one. This conclusion, I am sorry to say, was incorrect, or perhaps I may put it, rather, incomplete. *Helkesimastix* may possess, also, a very short, accessory flagellum, comparable with that shown by certain *Monas*.

1. Woodcock and Lapage, Proc. Roy. Soc. 83 B, p. 353.

In view of this necessary addition to our original description, I should like to point out that I have a definite recollection of observing *occasionally* — by no means often — a slight but distinct flickering, of a spasmodic character, at the right side of the body, near to the anterior end ; and in regard to this, Dr. Lapage concurs. But so frequently, when we had a promising individual before our eyes, gliding along quietly but steadily, we have not detected the slightest sign of movement at the anterior end, that we came to the conclusion that the impression we sometimes obtained was but the expression of a slight metabolic movement of the protoplasm in this region, similar to those commonly occurring in this Flagellate, as we have already described.

Recently, I happened to be watching an individual of *H. major* (the larger type, which we distinguished as a separate species), which had cropped up sparingly in an original culture, made for other purposes, when I caught sight clearly of a delicate, very short, curved flagellum-like process, projecting on the right side of the body, at the anterior end (text-fig. 1). It was most difficult to keep this in focus as the Flagellate travelled, even though it was gently gliding in one plane. Following this individual closely, I observed that the minute flagellum would be kept rigid and immovable for a period, and then would wave stiffly and somewhat slowly to the side of the body and back to its former position. Its behaviour is thus very different from that of the ordinary bending, vibratory motion of the anterior flagellum of *Bodo* or *Cercomonas*. Having once detected this delicate accessory flagellum, I knew exactly what to look for — a point which is of considerable assistance in the discernment of such minute organelles — and was able to see it in other individuals also ; but in spite of my efforts, I could not detect it in by any means all. I next obtained a culture of the original (smaller) form, *H. faecicola*, and succeeded in observing this flagellum in a proportion of the individuals in the case of this species also. Fortunately, Dr. Lapage happened to be in London at the time and he fully confirmed the discovery.

This much can certainly be said, in part extenuation of our failure to observe this accessory flagellum before. It is not of constant occurrence ; not present, that is, in all individuals. Its absence in some cases prevented us from verifying our tentative suspicions and led us to conclude there was only the long, trailing flagellum. Even when present, this second flagellum is most difficult to see. I have had this fact corro-

borated by noting the efforts of friends, experienced microscopists, to detect it when I have pointed it out to them.

Text-figs. 1-3 are from fixed and stained preparations. Figs. 1-2 were fixed by a special modification of Schaudinn's fluid, which I have used for several years and have found excellent for Protozoa generally. This modification was first described by me in 1916¹, and may be known as Woodcock's S. A. A. mixture. It consists of 2 parts of saturated aqueous solution of corrosive sublimate and one part of absolute alcohol (or rectified spirit), to which is added 5 p. 100 of glacial acetic acid. The stain used was Heidenhain's iron-haematoxylin (the long method). Try as one will, it is often most difficult to obtain preparations of these small, coprozoic Flagellates, in which the delicate flagella are well-stained. Sometimes, I have used an alcoholic solution of Eosin or Fuchsin, as a counter-stain. I have

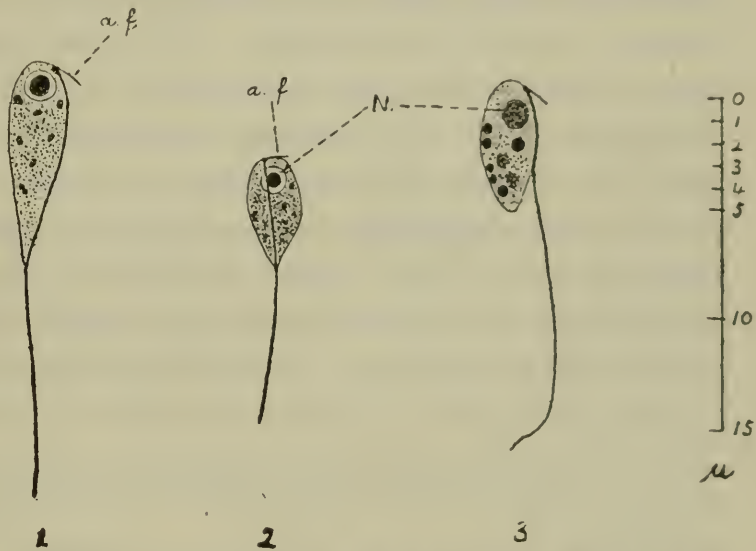


FIG. 1. *Helkesimastix major*. — FIG. 2. *H. faecicola*. — FIG. 3. *H. faecicola* stained by Giemsa (after osmic vapour). $\times 2250$.

found this method to give the best results, but even so, success is not invariably attained. If, however, my figures of *Spiromonas* (pl. 28, Phil. Trans., 1. c.) be compared with those of *Alphamonas*, recently described by Alexeieff, which I propose to consider in the following note, it will be seen that mine show the two flagella clearly, whereas in no case does Alexeieff figure both — sometimes, indeed, not even one.

Text-fig. 1 represents *Helkesimastix major*; fig. 2, *H. faecicola*; and fig. 3, the same form stained by Giemsa (after osmic vapour). The accessory flagellum cannot be detected in a large number of the individuals on the same films from which these figures were drawn. While it is probably present in some, nevertheless, I do not think that it is present in all, because of the fact that in fresh observation-preparations,

1. Phil. Trans. Roy. Soc., vol. 207 B, p. 379.

this second flagellum cannot be seen in the case of many individuals. Not only that, but I have a few heavily stained preparations, in which the long flagellum happens to be much more intensely stained than in either of the preparations from which figs. 1 and 2 were drawn, and yet in not a single individual can the accessory flagellum be seen. As stated above, therefore, this second flagellum is not constantly present, and in some races or strains appears to be altogether absent.

The accessory flagellum has a very definite orientation with regard to the body. When the Flagellate is gliding, the trailing flagellum always lies uppermost, and we may adopt the convention that, in this position, it is dorsal to the body. The small flagellum has its origin very close to that of the long one; there is a well-marked blepharoplastic thickening at the point of origin, but it is difficult to be certain whether there is a single large blepharoplast, or two in contact with each other. The accessory flagellum always projects slightly upwards (dorsally) and somewhat to the right side, never to the left. In life, it is best caught sight of as the creature swings slightly to and fro, about the axis of the trailing flagellum. As the Flagellate swings to the left, and the long flagellum passes a little to the right, the accessory flagellum projects outwards on that side, from the anterior end. In this position, its slow, waving motion can be observed. The fact that it is curved and not altogether in one plane adds to the difficulty of detecting it. Owing to its delicacy, at one focus its distal extremity only may be seen, appearing as a short rod or dot, travelling parallel to, but separate from the body. When at rest, this short flagellum is usually directed somewhat backwards, terminating about on a line with the middle of the nucleus. Occasionally, however, it projects straight outwards, at right angles to the long flagellum; in such a case, when the creature is lying in the true dorsi-ventral plane, the small flagellum gives a straighter, harder outline to the anterior end of the body (fig. 2). The length of this accessory flagellum varies from about $1u$ to $1\frac{1}{2}u$.

The presence of this accessory flagellum does not throw any light upon the peculiar gliding movement of *Helkesimastix*; because it is often held stiff and motionless while the creature is gliding forwards. It remains uncertain, in fact, what can be its function. It does not waft food-particles into the mouth, because there is no mouth; and we have never seen any indication of vacuolar ingestion and digestion of food, as occurs, for instance, in *Monas*. If *Helkesimastix* does ingest

solid particles, it does so at any part of its body, more particularly in the posterior region, in the same manner as does *Cercomonas*. There are frequently prominent, deeply staining, rounded grains in the cytoplasm (cf. fig. 3), but these are unlike the Bacteria, etc., in the surrounding medium and do not, I consider, represent ingested food. It is only necessary to compare the cytoplasm of, say, *Monas*, *Bodo*, or *Phyllomitus*, with its contained Bacteria and other food-particles, to see the difference. These large, round granules, represent, rather, some product of metabolic (probably nuclear) activity. But there can be no doubt, I think, that this small accessory flagellum is comparable with that of paramastigote types.

The nearest relationships of *Helkesimastix* are very difficult to determine. It resembles, in one character or another, several of these protomastigine forms and yet differs from them all in some important respect. On the whole, *Helkesimastix* is probably most nearly allied to *Cercomonas*, although there is the important difference that it has lost the powerful, long anterior flagellum of the latter type, but on the other hand has developed a short accessory flagellum, which *Cercomonas* does not possess.

2. *Alphamonas* Alexeieff a synonym of *Spiromonas* Perty.

ALEXIEFF recently described¹ a coprozoic Flagellate, which he regarded as an entirely new type and named *Alphamonas coprocola*, n. g., n. sp. The author considered that this form had claims to be considered intermediate (« forme de passage ») between Bacteria and Flagellates.

In comparing *Alphamonas* with certain other coprozoic Flagellates, Alexeieff considers — and quite rightly — that his form is not related to *Helkesimastix*. It is evident, however, that Alexeieff had not then seen my later paper on coprozoic Flagellates (ref. above), or he would certainly have realized that the form he describes is the same as that of whose life-cycle I there gave a much fuller account, namely *Spiromonas angustata* (Duj.)². I made all my original observations upon material derived from sheep- and goat-dung; but on noting that Alexeieff obtained his form from horse-dung, I started cultures of this material, and, as expected, have had no difficulty in obtaining *Spiromonas*.

1. *Arch. Zool. exp.*, vol. 57, 1918, N. et R. p. 1.

2. By an oversight, *angustata* is written *angusta* in my paper.

Alexeieff notes the occurrence of two flagella, although, as above mentioned, he does not figure an individual showing both. The author evidently had considerable difficulty in seeing the flagella. As I stated in my account, *Spiromonas* is one of the most active of coprozoic Flagellates, and when in rapid motion, it is impossible to detect the flagella. It is also, however, one of those which require most air, and in an ordinary cover-slip preparation (not an observation-preparation), the Flagellate very soon becomes languid and its movement ceases. As this happens, it is quite easy to observe both flagella; just as easy as it is to see the anterior, vibratile flagellum of *Bodo* and *Heteromita*, when these are at rest. While the two flagella of *Spiromonas* (or *Alphamonas*) are certainly more slender than the powerful anterior flagellum of *Cercomonas*, they are not so delicate and difficult to see as is the posterior, trailing flagellum of this type, when the posterior, end of the body is not drawn out along it. And the detection of the short accessory flagellum of *Helkesimastix* is a far greater test both of the lens and one's own acuity of vision! The sub-equal flagella of *Spiromonas* resemble most nearly those of *Bodo* (cf. *caudatus*) (*vide* my figures, pl. 27, l. c.). Alexeieff's description of the mode of movement of the body in *Alphamonas* agrees exactly with that of *Spiromonas*.

In regard to the size, the variation in size of the smaller forms, the characteristic spiral shape and general appearance, the remarkable, deeply staining mass in the posterior half of the body of the large forms, and the mode of multiplication, *Alphamonas* agrees completely with *Spiromonas*, and no more need be said upon these points. A comparison of my account and figures with those of Alexeieff settles the question.

In one or two respects, however, Alexeieff's description is incomplete or at fault. Division is not only quadri-partite. I have found that it is much more commonly tri-partite, three daughter-individuals being formed inside the cyst. Moreover, a definite cyst-membrane *is* present; this is very delicate, but unmistakeable (*vide* my fig. 65, pl. 28, l. c.). As mentioned in my account, sometimes the daughter-individuals, at the time of liberation, squirm out of the cyst successively, at the point where this is ruptured. In my own account I also pointed out that Martin and Lewin's fig. 37 related in all probability to *Spiromonas* and not to *Bodo caudatus*; and in their figure also, what appears to be the empty cyst is shown.

As regards the peculiar inclusion, which in stained preparations is

seen to consist of a mass of granules, I do not consider that this is in any sense a store of reserve food-material. It is not made use of in any way by the daughter-individuals. I think it is purely an adaptation to multiplication within a cyst. It must be remembered that these cysts are not resting, resistant cysts, but for multiplication in the same medium and under the same conditions in which the Flagellate at the moment happens to be. There is no alteration in the toxicity of the environment, no development of fresh ferments to dissolve the cyst-membrane, as when resistant cysts are placed in fresh medium (or become moistened with fresh water). There must be some other means of causing the rupture of the cyst-membrane. As indicated in my paper, I consider that when the formation of the daughter-individuals is completed and the granular mass cut off from the living protoplasm, it is able suddenly to absorb water from the surrounding medium, a large proportion of the mass being at the same time dissolved, and the expansion produced by this intake of water bursts the cyst. I generally noted that, immediately before liberation, the whole contents of the cyst seem to swell up and the outlines of the individuals become momentarily indistinct.

I am quite unable to agree with Alexeieff's suggestion that *Spiromonas* may perhaps be regarded as an intermediate or transitional form between the Bacteria and the Flagellates. In my opinion it is a true Flagellate and has no closer relationship to Bacteria than has any other Protomastigine Flagellate. In the first place — and, perhaps, the most important point — the flagella are typical Flagellate flagella, and not in the least comparable with bacterial flagella (or cilia). They are long, conspicuous in life, and stainable by the usual cytological methods; moreover, they *do* originate from a blepharoplast (cf. my figs. 20-25, pl. 27, l. c. from Giemsa stained smears). (In preparations stained with iron-haematoxylin, the blepharoplast cannot by any means always be detected in connection with the delicate flagella of these minute Flagellates.) In all these characters, the flagella of *Spiromonas* differ from those of *Spirilla*.

Again, the body is not of fixed, rigid shape. While it is not amoeboid, or « metabolic » as in the case of certain coprozoic forms (*e. g.* *Cercomonas*, *Monas*), it nevertheless alters in shape decidedly as it grows, becoming, as I described, ovoid or bean-like. There is no resemblance between the mode of increase in size here and that in the case of Bacteria. Further, the nucleus is a definitely constituted organella. Its diffe-

rentiation into nuclear membrane, linin-reticulum, and karyosome marks a great advance on any type of nuclear structure found in Bacteria. In the case of many nuclei of the protokaryon type, commonly found in these simple Flagellates, practically all the chromatin is contained in the karyosome ; *Spiromonas* is no exception in this respect (cf. my figs. of *Bodo*, *Heteromita*, *Phyllomitus*, etc.).

As regards the life-cycle, I have shown above that there is really nothing comparable with « epiplasm » in the multiplicative cyst of *Spiromonas*. The division into 3 or 4 daughter-individuals within a delicate ectocyst cannot be correctly compared with endosporous spore-formation in certain Bacteria or Saccharomycetes. It is essentially an *ectosporous* process-not the formation of daughter-individuals or spores *within* the body of the parent. It is much more comparable, for instance, with the schizogony of Telosporidia (Ectospora)¹ than with the spore-formation in Neosporidia (Endospora). Lastly, conjugation (syngamy) occurs in *Spiromonas*, as I have found to be the case in many other of these coprozoïc Flagellates ; and up to the present no evidence is forthcoming of any such phase in the life-cycle of Bacteria.

Spiromonas has no connection with *Ancyromonas*. It is undoubtedly a Heteromastigine type, and best placed, I consider, in the Fam. *Bodonidae*. Its nearest relationships are probably with *Bodo* and *Heteromita* ; but it differs from these forms in that the posterior flagellum never appears to function as a passive trailing flagellum and also in regard to certain phases in the life-cycle.

1. In certain Haemogregarines, schizogony takes place inside a delicate cyst-membrane.

XIX. THE ACTION OF YEAST-GROWTH STIMULANT.

BY OSWALD KENTISH WRIGHT.

From the Biochemical Department, Lister Institute, and the Bacteriological Laboratory, Household and Social Science Department, King's College for Women.

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WILLIAMS [1919] and Bachman [1919] have confirmed the observation of Wildiers [1901] that certain yeasts are only able to grow at the expense of ammonium salts provided that a heavy inoculation is employed or a small amount of organic material (termed "bios" by Wildiers) is added to the medium. After a study of the ability of various substances to produce this effect, they suggest that the essential substance, which we will continue to call "bios" for the sake of convenience, is identical with the water-soluble *B* or anti-beri-beri vitamin. Williams [1920] has gone further, and has elaborated a method for measuring quantitatively the amount of anti-beri-beri vitamin present in any substance by observing its effect on the growth of yeast.

This suggestion has given rise to much discussion [see Eddy, 1921] and at present it is generally considered that the case for the identity of the two principles is not proven.

Lemon juice freed from citric acid by the method of Harden and Zilva [1918] when added to a mineral nutrient solution in small quantities enables a yeast to grow which could not grow in its absence. As animal feeding experiments show that the amount of water soluble *B* vitamin in lemon juice is very small relative to that in yeast extract [see Osborne and Mendel, 1920], it was thought that an investigation of its effect on the growth of yeast in mineral nutrient solutions might throw some light on the way in which the stimulation is brought about.

Preliminary experiments were performed to ascertain the smallest amount of lemon juice capable of producing growth in a nutrient solution made up as follows (Williams):

Saccharose	...	20 g.
(NH ₄) ₂ SO ₄	...	3 g.
KH ₂ PO ₄	...	2 g.
CaCl ₂	...	0.25 g.
MgSO ₄	...	0.25 g.
Distilled water	...	1000 cc.

A series of tubes was prepared containing this solution with the addition of increasing percentages of lemon juice. The yeast employed was a pure

culture of a baker's yeast isolated by Dr Harden. This was grown for 24 hours in yeast water glucose and the growth was washed three times with sterile distilled water by means of the centrifuge. A loopful of a suspension of this washed yeast was put into each tube of the series, the amount being judged so that a fair proportion of drops made, according to Lindner's method, with a mapping pen on a cover slip contained one, two, or three cells each. The coverslip was sealed over a moist chamber with vaseline and, after noting the number of cells in each drop, was incubated for 24 hours at 25° and again examined for growth.

By this method it was found that no growth took place in the mineral nutrient solution unless 5 % or more of lemon juice was added. It was also noticed however that some of the tubes containing smaller amounts of lemon juice than 5 %, which had been standing in the laboratory, contained an amount of yeast appreciably larger than the amount originally inoculated. This suggested that growth did not take place as readily in the case of one or two cells in a minute drop as in a larger amount of medium. Subsequent investigation confirmed this, and the drop method was abandoned in favour of estimating the number of cells per cc. by means of a counting chamber.

In order to avoid adding any appreciable amount of "bios" with the seed yeast, it is necessary to inoculate each tube with as small a quantity of yeast as possible. Attempts to inoculate tubes by picking up a drop of distilled water containing one, two, or three cells from a coverslip by means of sterile filter paper proved unsuccessful. The method adopted was to make a light emulsion in sterile distilled water of yeast cells prepared and washed as described above. A slide was marked with several circles with a grease pencil and a drop of the emulsion transferred to the centre of each by means of a platinum loop. The drops were dried and stained and the number of cells in each counted, and by this means an emulsion was prepared which contained somewhere between 50 and 500 cells in a loopful. One loopful was then used to inoculate each tube.

A series of ten tubes was prepared with a mineral nutrient solution containing $(\text{NH}_4)_2\text{SO}_4$ with increasing percentages of lemon juice. A similar series was prepared without the $(\text{NH}_4)_2\text{SO}_4$.

Each tube contained 7 cc. of the following solution (Williams' solution without the $(\text{NH}_4)_2\text{SO}_4$, see p. 137):

Saccharose	...	20 g.
KH_2PO_4	...	2 g.
CaCl_2	...	0.25 g.
MgSO_4	...	0.25 g.
Distilled water	...	1000 cc.

The tubes containing $(\text{NH}_4)_2\text{SO}_4$ received 1.5 cc. of a solution containing 2 g. of the salt in 100 cc. The saccharose and the $(\text{NH}_4)_2\text{SO}_4$ were purified by several recrystallisations. Repeated attempts to grow the yeast in tubes of this solution with $(\text{NH}_4)_2\text{SO}_4$ inoculated with from 50 to 500 cells in the

manner described above and without the addition of "bios" failed, no growth being observed even after a lapse of several weeks.

The lemon juice was prepared by the method of Harden and Zilva [1918], namely as follows: calcium carbonate was stirred into fresh lemon juice in excess of the amount required to neutralise all free acid. Absolute alcohol to twice the volume of the original lemon juice was added, and the whole filtered. The filtrate was evaporated down almost to dryness *in vacuo* at 35°, and the residue made up to the original volume of the lemon juice with distilled water. The sample used contained 0.0868 mg. of nitrogen per cc. of which 0.0112 mg. was ammoniacal nitrogen. The desired amount of this lemon juice was added to each tube of the series which was then made up to 10 cc. with distilled water. The tubes were sterilised by steaming for 30 minutes on each of three successive days. Each was then inoculated with from 50 to 500 cells as described above, the tubes were aerated by blowing air into them through a sterile glass tube plugged with wool and having its end drawn out to a fine point, and were incubated at 25°.

The tubes were examined each day and the number of cells counted by means of a Thoma haemocytometer. At least four drops were counted from each tube and if any considerable discrepancy was found between them the counting was continued until the margin of error was reduced below 5%. Owing to the number of tubes it was not possible to count all of them every day, but the different rates at which the yeast grew in the various tubes rendered this unnecessary. The results are set out in Table I.

Table I.

Number of tube	Percentage of lemon juice	(NH ₄) ₂ SO ₄	Days: growth in millions of cells per cc.						
			1	2	3	4	5	6	9
1	0.1	0	s.g.	s.g.	s.g.	s.g.	—	s.g.	1.6
11	0.1	+	"	"	"	"	—	1.5*	29.0†
2	0.25	0	"	"	"	"	—	1.8	1.0
12	0.25	+	"	"	"	"	—	4.3*	32.6†
3	0.5	0	"	"	"	1.3	—	3.5	1.0
13	0.5	+	"	"	"	1.7	—	2.1*	34.2†
4	0.75	0	"	"	"	0.9	—	2.5	1.8
14	0.75	+	"	"	"	1.0	—	4.9*	26.7†
5	1.0	0	"	"	2.3	3.1	—	2.7	6.1
15	1.0	+	"	"	1.1	1.4	—	4.6*	22.6†
6	2.5	0	"	2.8	4.3	—	6.3	—	—
16	2.5	+	"	3.1	5.5	—	21.2	—	—
7	5.0	0	"	4.9	7.1	—	—	—	—
17	5.0	+	"	4.8	27.4	—	—	—	—
8	7.5	0	"	6.7	9.2	—	—	—	—
18	7.5	+	"	21.1	19.4	—	—	—	—
9	10.0	0	"	12.0	12.1	—	—	—	—
19	10.0	+	"	26.5	29.4	—	—	—	—
10	15.0	0	"	15.9	19.6	—	—	—	—
20	15.0	+	"	24.3	40.6	—	—	—	—

S.g. means some growth but too slight to count.

* Involution forms present.

† Films formed. Accurate counting impossible owing to clumping of cells. Many involution forms.

It is well known that the growth of yeast is susceptible to slight variations of conditions which cannot be controlled absolutely, such as the degree of aeration and the formation of products of metabolism and fermentation. Further, the accuracy of the counting is affected by the smallness of the drop examined in the low counts and by the tendency of the cells to form clumps in the higher counts or in the later stages of growth, so that below 3,000,000 and above 15,000,000 cells per cc. the counts of different drops of the same sample are found to be noticeably less uniform than between those figures, and the counts made after more than six days of growth can only be regarded as approximate. In considering the results obtained regard must be paid to these facts, and account taken only of large differences in the numbers. The table shows, however, that, until the yeast reaches a concentration of somewhere in the neighbourhood of five or six million cells per cc., the rate of growth is independent of the presence or absence of $(\text{NH}_4)_2\text{SO}_4$ and depends on the concentration of the lemon juice.

It does not appear unreasonable that it should take longer for an individual cell to collect sufficient nutriment to enable it to multiply when the nutriment is dilute than when it is concentrated, and possibly the fact that cells were unable to multiply in a minute drop in dilutions that enabled growth to take place in larger quantities of medium is explained by the fact that the whole drop did not contain sufficient nutriment.

After the yeast reaches a concentration of five or six million cells per cc. it is able to continue growing freely in the $(\text{NH}_4)_2\text{SO}_4$ tubes. Further it was observed that after six days involution forms, as described by Will [1895] in his investigation of film formation, begin to appear in all tubes containing $(\text{NH}_4)_2\text{SO}_4$ where the growth had not reached the critical point of five or six million cells per cc., and by the ninth day there was a heavy growth of film yeast on all these tubes. All the remaining tubes after a lapse of three weeks showed only very rare involution forms and no film formation, but most of the cells were highly refractile with many large vacuoles and granules and a thick membrane, similar in appearance to the permanent cells described by Will. It would appear, then, that after an interval of six or seven days the cells in smaller concentrations than five or six million per cc. are able to adapt themselves to the use of $(\text{NH}_4)_2\text{SO}_4$ and that film formation results.

A similar series of tubes was prepared with increasing percentages of aqueous yeast extract instead of lemon juice. This was prepared by Osborne and Wakeman's method [1919]; about 250 g. of wet brewer's yeast was washed and boiled for two minutes with 1 litre of distilled water containing 0.01 % of acetic acid and the liquid was separated off in the centrifuge. The solid residue was again heated with 500 cc. of 0.01 % acetic acid and the extracts united and concentrated to 500 cc. This extract contained 2.856 mg. of nitrogen per cc., of which 0.2273 was ammoniacal nitrogen. As this is rather more than 30 times the amount contained in the lemon juice, the amounts added to the various tubes of the yeast extract series were much smaller than in the lemon juice series. The results are set out in Table II.

Table II.

Number of tube	Percentage of yeast extract	$(\text{NH}_4)_2\text{SO}_4$	Days: growth in millions of cells per cc.						
			1	2	3	4	5	6	11
21	0.01	0	s.g.	s.g.	s.g.	s.g.	—	—	3.1
31	0.01	+	"	"	"	"	—	—	10.6*
22	0.02	0	"	"	"	"	—	—	3.0
32	0.02	+	"	"	"	"	—	—	10.5*
23	0.05	0	"	"	"	"	—	—	4.0
33	0.05	+	"	"	"	"	—	—	15.0*
24	0.1	0	"	"	"	"	—	1.6	10.0*
34	0.1	+	"	"	"	"	—	15.1*	20.0*
25	0.15	0	"	"	"	6.1*	—	10.0*	—
35	0.15	+	"	"	"	3.6	—	11.5*	—
26	0.2	0	"	"	0.5	3.6	5.1	—	—
36	0.2	+	"	"	0.5	3.7	15.5	—	—
27	0.5	0	"	4.9	7.6	—	—	—	—
37	0.5	+	"	4.4	23.7	—	—	—	—
28	1.0	0	"	5.3	14.5	—	—	—	—
38	1.0	+	"	11.8	39.5	—	—	—	—
29	2.0	0	"	13.6	27.2	—	—	—	—
39	2.0	+	"	19.9	43.0	—	—	—	—
30	3.0	0	"	} Very heavy growth. Not counted.					
40	3.0	+	"						

S.g. means some growth but too slight to count.

* Involution forms present. Very slight film formation.

On comparing the results obtained with the yeast extract with those obtained with the lemon juice it will be seen that their general trend is similar, and that the yeast extract is approximately ten times as effective as the lemon juice although its nitrogen content is more than 30 times as great. The results obtained are somewhat less uniform than those obtained with the lemon juice, and tubes Nos. 24 and 25 fall out of line on the 11th and 4th days respectively. Film formation does not occur so readily, only very slight traces being present in tubes Nos. 31, 32, 33, 34 and 35 by the 11th day.

These differences may be due to differences in the nature of the nitrogen in the two substances under examination or to the presence of toxic substances in the yeast extract which are absent from the lemon juice. The yeast experiment, however, affords confirmation of the fact that the rate of growth is at first independent of the presence or absence of $(\text{NH}_4)_2\text{SO}_4$ and depends on the concentration of the "bios" until the yeast has reached a concentration of somewhere in the neighbourhood of five or six million cells per cc., after which it proceeds further in the presence of the $(\text{NH}_4)_2\text{SO}_4$.

GENERAL CONCLUSIONS.

The suggestion that "bios" may be of the same nature as the vitamins, which are widely held to be necessary for the nutrition of higher animals, and the further suggestion that it may be identical with one of the recognised vitamins, open up possibilities for investigating the subject of vitamins which are particularly attractive, owing to the ease with which a biological process

can be studied in a yeast as compared with a similar study in the case of a more complex organism. Before we can proceed to investigate the general question of vitamins by such methods it is necessary, firstly, to be certain that "bios" is actually a vitamin, *i.e.* a substance whose presence in the food in relatively small quantities is necessary to enable the remainder of the foodstuffs to be properly assimilated and utilised by the organism, and secondly that the substance in question is identical with one of the recognised vitamins. The present investigation points to the fact that "bios" does not enable the yeast to assimilate $(\text{NH}_4)_2\text{SO}_4$ simply by its presence or by being consumed at the same time, but that the yeast grows solely at the expense of the "bios" until it reaches a certain degree of concentration, and after that it is able to use the $(\text{NH}_4)_2\text{SO}_4$. No explanation is offered of this phenomenon which requires further investigation.

I wish to thank Prof. A. Harden, who suggested the investigation, for his advice and assistance in carrying it out.

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